AD_____

Award Number: DAMD17-99-1-9378

TITLE: A Novel Gene Delivery System Targeted to Breast Cancer Cells

PRINCIPAL INVESTIGATOR: Laurence H. Kedes, M.D.
Lali K. Medina-Kauwe, Ph.D.

CONTRACTING ORGANIZATION: University of Southern California
Los Angeles, California 90023

REPORT DATE: August 2002

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 074-0188*



| 1. AGENCY USE ONLY (Leave blank) | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | August 2002 | Final (1 Aug 99 - 31 Jul 02) |

**4. TITLE AND SUBTITLE**
A Novel Gene Delivery System Targeted to Breast Cancer Cells

**5. FUNDING NUMBERS**
DAMD17-99-1-9378

**6. AUTHOR(S)**
Laurence H. Kedes, M.D.
Lali K. Medina-Kauwe, Ph.D.

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
University of Southern California
Los Angeles, California 90023

E*Mail: kedes@hsc.usc.edu

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSORING / MONITORING AGENCY REPORT NUMBER**

**11. SUPPLEMENTARY NOTES**
report contains color

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

**12b. DISTRIBUTION CODE**

**13. Abstract** ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

We are producing a gene delivery vehicle that uses unique features of viral proteins in a targeted, non-viral gene delivery system. The key features of this system that will lend themselves to gene therapy are: 1) the missile-like targeting capacity; 2) the capacity to transport DNA, and therefore, therapeutic genes; 3) the lack of size restriction, and therefore, the potential capacity to carry any size gene; 4) the lack of viral genes, and therefore, virulence; and, 5) the enhanced ability to enter cells and deliver therapeutic genes. While we expect this system to retain some of the best properties of viral vectors while taking advantage of mechanisms that viruses have evolved to efficiently enter cells, we also expect this system to improve on these mechanisms by introducing cell type-specific targeting capabilities. Because this system is non-viral in nature, it avoids problems inherent in biologic agents, thus enabling pharmaceutical quality control over the final preparations. Potential application of this system includes, but is not limited to, the targeted delivery of therapeutic genes or drugs for cancer treatment or gene replacement therapy.

To validate our system, we are incorporating the targeting ligand, heregulin, which binds with high affinity to certain human breast cancer cells, and the herpes simplex thymidine kinase gene as our therapeutic gene payload. This system will be tested on human breast cancer cells in culture and in animal tumor models in order to assess its efficacy both *in vitro* and *in vivo*.

| 14. SUBJECT TERMS | 15. NUMBER OF PAGES |
|---|---|
| breast cancer | 38 |
| | 16. PRICE CODE |

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | Unlimited |

## Table of Contents

## INTRODUCTION

The subject of this research is to create a novel gene delivery system that makes use of recombinant adenoviral capsid protein components engineered to deliver genes to breast cancer cells in the absence of both a viral genome and the expression of other adenoviral proteins.

The purpose of this novel system is to deliver therapeutic genes in a ligand-directed, cell type-specific manner, thus avoiding normal cells and blood cells and therefore enhancing the safety of breast cancer therapy.

The scope of the research is to characterize the functional properties of this gene delivery system, and test its targeting ability and efficacy on both cells in culture and *in vivo*.

## BODY

*Task 1.* To characterize the functional properties of recombinant fibers that contain targeting ligand sequences, recombinant pentons that contain polylysine tracts for DNA binding, and assembly of recombinant fiber-penton complexes.

Production and assembly of recombinant penton and fiber proteins. We used a bacterial protein expression system to produce the recombinant penton protein, PBK10, and have shown previously that this protein can pentamerize in vitro (Medina-Kauwe et al., 2001a; See **Appendix 1**).

E. coli transformed with the Ad5 fiber gene exhibits severely attenuated growth and negligible levels of recombinant protein expression. The fiber knob domain, on the other hand, can be produced at high levels from E.coli, suggesting that the fiber shaft is toxic to bacteria. We used a bacterial protein expression system to produce recombinant knob protein, and have shown previously that this protein can trimerize *in vitro* (Medina-Kauwe et al., 2000; See **Appendix 2**). Meanwhile, we re-cloned the Ad5 fiber gene into a baculovirus transfer plasmid that encodes glutathione S-transferase (GST) just N-terminal to the fiber to enable glutathione-immobilized affinity purification. The fusion protein, GST-WTF, can be detected in infected cell lysates (**Fig. 1a, lane 2**). Protease cleavage at an enterokinase (EK) site located between the GST and fiber sequences liberates full-length, "fusionless" wild-type fiber (WTF) protein of the expected molecular weight of about 64 kilodaltons (kDa) (**Fig. 1a, lane 3**). Protease treatment of GST-WTF bound to glutathione resin followed by removal of EK yields a purified protein (**Fig. 1b, lane 1**). We consistently find that our methods yield a purified doublet, both bands of which are recognized by an anti-Ad5 capsid protein polyclonal antibody, thus verifying their identities (**Fig. 1c**). As the fiber peptide sequence contains no other EK sites, this doublet may be due to glycosylation in insect cells, as reported elsewhere [6]. Under non-denaturing conditions, the recombinant WTF molecular weight is about 190 kDa, consistent with the formation of trimers (**Fig. 1d**), and interacts with the recombinant penton, PBK10 (**Fig. 2a**). Although notable levels of "fusionless" GST are produced prior to the protease digest (**Fig. 1a, lane 2**), this protein is removed from the final preparation by performing the digest while the proteins are bound to glutathione resin, thus yielding purified protein (**Fig. 1b, lane 1**).

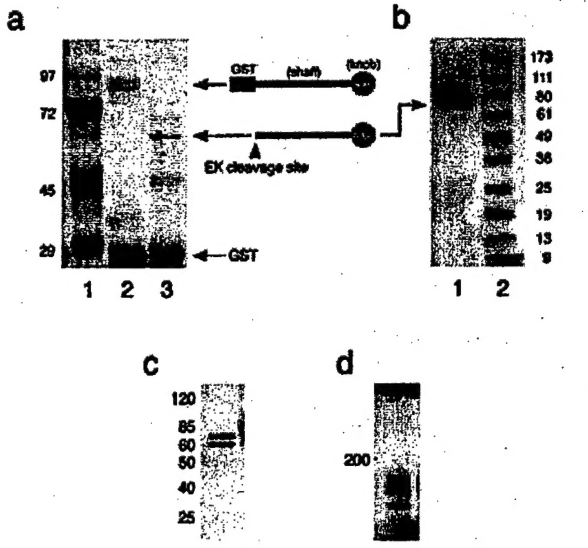


**Figure 1.** Production, purification and testing of recombinant fiber. (a) Coomassie-stained protein gel containing a high molecular weight protein marker (lane 1), and GST-WTF pre- (lane 2), and post- protease cleavage (lane 3) after electrophoresis under denaturing conditions. (b) Coomassie-stained denaturing gel containing purified WTF (lane 1) and molecular weight protein marker (lane 2). (c) Western blot of WTF after electrophoresis under denaturing conditions. Arrows point to protein doublet. (d) Western blot of WTF after non-denaturing gel electrophoresis. Trimer is indicated by a "t". The numbers to the sides of the figures denote the sizes of the molecular weight marker bands.

Production of the targeted fiber, FVL-Her. The remaining goal to be met for Task 1 is the production of a targeted full-length fiber that can be assembled with PBK10. This goal has now been met by the expression and purification of the targeted fiber protein, FVL-Her. This protein contains, from the amino (N) to the carboxy (C) terminus respectively, the Ad5 fiber tail and shaft domain followed by a trimerization region (VL) derived from the Moloney murine leukemia virus (MMLV) envelope, and the targeting ligand, Her (see **Fig. 2**). The

fiber tail binds to the Ad5 penton base. The VL region self-trimerizes and is intended to promote trimerization of the targeted fiber. The targeting ligand, Her, is the receptor binding domain of the ligand, heregulin, which binds with high affinity to HER2 receptors which are overexpressed on many types of breast cancers.

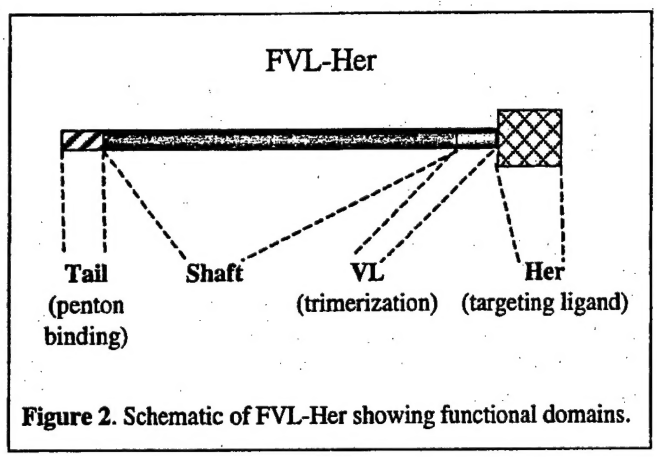


**Figure 2.** Schematic of FVL-Her showing functional domains.

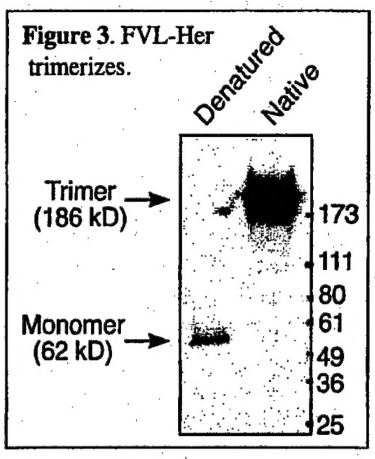


**Figure 3.** FVL-Her trimerizes.

FVL-Her trimerizes. As the natural quaternary structure of the wild-type fiber is a trimer, it is highly likely that retaining trimerization may aid in assembly with pentons. The trimerization ability of FVL-Her was tested by determining the size of oligomers formed under non-denaturing conditions. The expected sizes of FVL-Her monomer and trimer proteins are 62 and 186 kilodaltons (kD), respectively. Both the denatured and native proteins migrate according to these predicted molecular weights, as determined by polyacrylamide gel electrophoresis (PAGE) analysis (**Fig. 3**).

FVL-Her binds heregulin receptors. To determine whether FVL-Her binds breast cancer cells specifically through the heregulin receptor, we used an established assay that detects the competitive inhibition of soluble recombinant GFP-tagged ligands bound to their respective receptors (Medina-Kauwe et al., 2000). We have shown previously that receptor binding of a GFP-heregulin fusion protein (GFP-Her) to MDA-MB-453 human breast cancer cells can be specifically inhibited by increasing concentrations of free Her protein. Using fluorescence activated cell sorting (FACS) analysis, we show that a 10x molar excess of either Her or FVL-Her reduces GFP-Her receptor binding by 70-80% or 50-60%, respectively (**Fig. 4**). In contrast, free knob protein has little to no effect on heregulin receptor binding, despite the presence of both CAR and heregulin receptors on MDA-MB-453 cells (Medina-Kauwe et al., 2000). Both GFP-Her and FVL-Her contain the same heregulin domain as C-terminal fusions. This data, thus, suggests a likelihood of specificity in the design of FVL-Her targeted Ad, and furthermore shows that the receptor binding capacity of FVL-Her is changed little from the wild-type ligand.

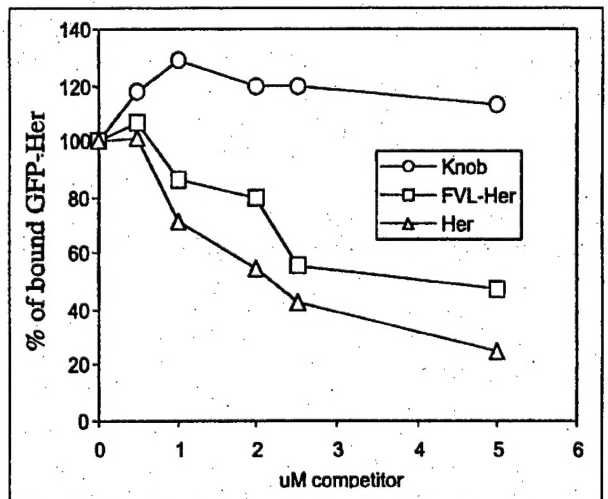


**Figure 4.** Binding specificity of FVL-Her. Graph summarizes the FACS analyses of MDA-MB-453 cells bound with 0.5 uM GFP-Her and the indicated concentrations of competitors.

Cellular effects of heregulin receptor binding. We have consistently observed a morphological change in the appearance of MDA-MB-453 human breast cancer cells in culture after exposure to free soluble Her. A 0.9uM concentration of Her induces many of the cells to assume a flatter more differentiated appearance after 6 hours of exposure (**Fig. 5**). This effect appears to be responsive to Her concentration and exposure time to the cells, and is reminiscent of earlier studies showing the induction of MDA-MB-453 cell differentiation after exposure to HepG2 cell conditioned medium (Plowman et al., 1993a & b). The unidentified factor present in the conditioned medium in these studies specifically stimulated the tyrosine kinase activity of the HER4 receptor subunit (*HER4/erbB4*), but not EGF-R, HER2, or HER3, which are also present on MDA-MB-453 cells. Later studies showed that heregulin induces the tyrosine phosphorylation of both HER3 and HER4 receptor subunits in MDA-MB-453 cells in a time and dose dependent manner (Sepp-Lorenzino et al., 1996), in association with high affinity binding to HER4 but low affinity binding to HER3 (Plowman et al., 1993a & b; Carraway et al., 1994a & b; Kita et al., 1994; Sliwkowski et al., 1994). Although heregulin does not directly interact with HER2, the cellular response to heregulin binding depends on the level of HER2 expression. Cell lines that overexpress HER2, such as MDA-MB-453 cells, proliferate in response to relatively low levels of heregulin but

undergo mitogenic arrest in response to higher levels (Bacus et al., 1992; Lupu et al., 1992; Peles et al., 1992). Conversely, cell lines that express low levels of HER2 proliferate in response to any concentration of heregulin (Lupu et al., 1995).

*Task 2.* To test the ability of recombinant fiber-penton complexes to target delivery of: (a) the ß-galactosidase (lacZ) marker gene, and (b) the herpes simplex virus thymidine kinase (HSV-tk) gene, specifically to human breast cancer cells in cell culture.

Reporter genes encoding the green fluorescent protein (GFP) and luciferase (Luc) were used in place of the lacZ marker gene. Monitoring GFP expression is a convenient way to detect gene delivery in living cells and allows the user to measure expression over time. Assays measuring luciferase activity are well-established and instrumentation for detecting this activity has made this technology convenient for rapid analysis and high throughput. For these reasons, GFP and Luc, instead of lacZ, have been used here to monitor gene delivery.

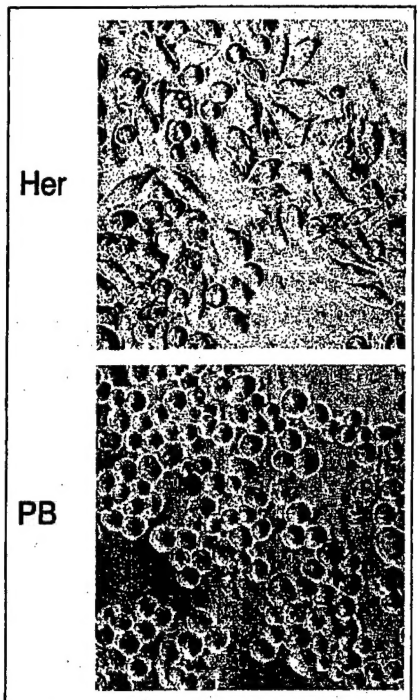


**Figure 5.** Morphological effect of Her treatment of MDA-MB-453 cells. Cells were exposed to 1 ug of Her (A) or the Ad5 penton protein, PB, (B) for 6 hours. PB treated cells are morphologically unchanged from untreated cells (not shown).

3PO, a novel non-viral gene delivery system using engineered Ad5 penton proteins. We have previously demonstrated that the Adenovirus serotype 5 (Ad5) capsid penton protein, which is responsible for Ad5 cell binding and entry during viral infection, can be engineered to assemble with plasmid DNA and deliver a reporter gene into cultured cells in the absence of whole virus (Medina-Kauwe et al., 2001a; See **Appendix 1**). The engineered penton, called PBK10, gained the function of DNA binding by recombinant fusion to a polylysine sequence. The addition of protamine, a small polycationic peptide, to penton-bound DNA produced complexes named 3PO, and allowed gene delivery in the presence of serum. As the Ad5 penton binds $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins through a conserved Arg-Gly-Asp (RGD) consensus motif (Goldman and Wilson, 1995; Mathias et al., 1994; Neumann et al., 1988), the ability of RGD-containing oligopeptides to inhibit 3PO-mediated gene delivery helped to confirm its cell binding specificity. By exploiting the integrin binding and endocytosis pathway typically used by Ad5, we have shown that cell entry of exogenous genes likewise may be accomplished using the Ad5 penton only. The endosomal penetration and cytoplasmic entry features of Ad5 are also attributed to the penton (Karayan et al., 1997; Seth et al., 1984) and are thus useful here for trafficking of exogenous DNA into the cytoplasm. Luciferase reporter gene activity produced by 3PO-mediated gene transfer was up to 115-fold and 190-fold higher than that produced by protamine + DNA (PO) alone in HeLa and 293 cells, respectively (293 cells not shown) (**Fig. 6A**). PBK10-mediated delivery of a green fluorescent protein (GFP) gene produced up to 65% GFP-positive cells (**Fig. 6B**).

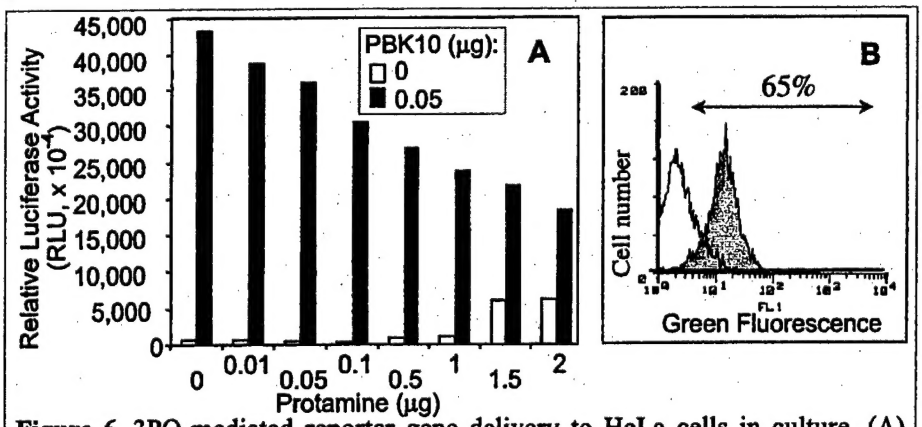


**Figure 6.** 3PO-mediated reporter gene delivery to HeLa cells in culture. (A) Luciferase activity of cells transduced by 3PO (filled bars) or PO (open bars). 0.1 µg of DNA used. (B) FACS analysis of GFP-positive cells (shaded) transduced by PBK10-mediated gene delivery. Untreated cells are represented by an open histogram.

Non-viral gene delivery to human breast cancer cells by targeted Ad5 penton proteins. Based upon our previously described system, we determined whether penton-based gene delivery can be targeted to receptors other than $\alpha_v$ integrins. To test this, we produced an adenovirus penton fusion protein, HerPBK10, which contains the EGF-like domain of the heregulin-$\alpha_1$ ligand as an amino (N) –terminal fusion to PBK10 (Medina-Kauwe et al., 2001b; See **Appendix 3**). This ligand binds with high affinity to HER2/3 or HER2/4 heterodimers, which are overexpressed on certain aggressive breast cancers and human breast cancer cell lines,

including MDA-MB-453 cells (Bacus et al., 1994; Carraway and Burden, 1995; Carraway and Cantley, 1994; Carraway et al., 1994; Goldman et al., 1990; Hung et al., 1995; Press et al., 1990; Slamon and Clark, 1988; Slamon et al., 1987; Yarden and Weinberg, 1989). In addition, we and others have shown that this ligand is rapidly internalized after binding, thus adding to the utility of heregulin for targeting (Lenferink et al., 1998; Li et al., 1996; Medina-Kauwe, 2000; Waterman et al., 1998). HerPBK10 binds MDA-MB-453 human breast cancer cells in a receptor-specific manner, and mediated the entry of a reporter plasmid in MDA-MB-453 cells in culture. Excess heregulin peptide competitively inhibited HerPBK10-mediated gene delivery, thus confirming receptor-specificity. Importantly, the penton segment appeared to contribute significantly to enhanced delivery, as fusion proteins lacking the penton domain exhibited a 97% reduction in gene transfer. The penton domain of HerPBK10 did not participate in binding to integrin receptors, as excess integrin-specific blocking peptides had a low to modest inhibition of gene delivery. Thus, it appeared that the penton was required for intracellular steps, such as endosomal lysis, after internalization.

Gene delivery complexes made between HerPBK10, protamine and plasmid DNA were named H2PO and, like 3PO, could mediate gene transfer *in vitro* in the presence of up to 10% serum, suggesting a potential for *in vivo* delivery. The endosomolytic agent, chloroquine, did not affect H2PO-mediated transduction, suggesting that the complexes were not trapped in endosomes after receptor-mediated endocytosis. However, whereas 95% of MDA-MB-453 cells were specifically bound by HerPBK10, only 6% showed reporter gene expression. A post cytosolic entry step, therefore, may account for the observed difference between HerPBK10 receptor binding and final gene expression by H2PO.

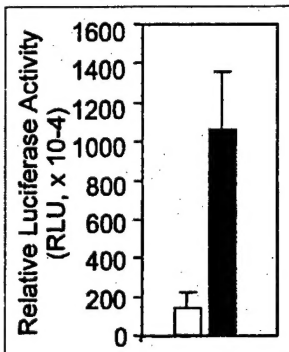


**Figure 7.** Luciferase activity of Hela cell lysates transduced with either 3PO (open bar) or 3POF (filled bar) delivering 0.1 µg of plasmid DNA.

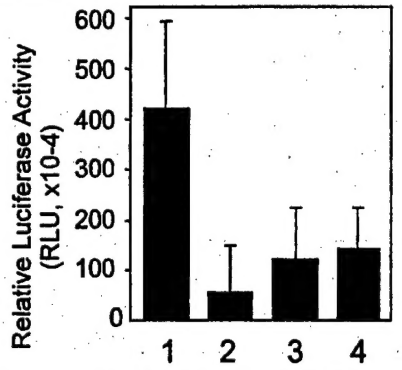


**Figure 8.** Inhibition of 3POF-mediated luciferase gene delivery by free knob. Bars denote luciferase activity encoded by 0.1 µg of pGL3 plasmid DNA delivered by 3POF in the (1) absence of knob, or in the presence of (2) 1, (3) 10, (4) and 100-fold molar excess of knob protein over fiber concentration.

The fiber enhances 3PO transduction by binding to fiber receptors. WTF was incubated with 3PO to allow assembly of fiber-penton capsomers with the DNA complexes. The new complexes, named 3POF, were tested for delivery and expression of a luciferase gene by incubation with HeLa cells in culture. HeLa cells express both CAR and $\alpha_v$ integrins, and are infectable by Ad5. Luciferase activity following 3POF exposure is 8-fold higher than 3PO, suggesting higher gene delivery in the presence of the fiber (**Fig. 7**). The specificity of gene delivery by 3POF was confirmed by the demonstration that soluble knob protein competitively inhibits gene delivery and expression. We and others have shown that the knob protein can form trimers in the absence of the rest of the fiber, and can bind fiber receptors on cultured cells [18]. Here we show that soluble knob protein reduces 3POF-mediated gene delivery and expression in cultured HeLa cells by up to 87%, indicating that 3POF-mediated gene delivery requires binding mainly to CAR (**Fig. 8**).

F3PO is less cytotoxic *in vitro* than adenovirus. The cytotoxicity of F3PO and its individual components was tested on Hela cells in culture using an established assay that determines the relative numbers of living cells by measuring metabolic conversion (CellTiter, Promega Corporation, Madison, WI, USA). A concentration of F3PO resulting in 3.1 x $10^4$ pentons per cell, as well as the individual protein components, promoted undetectable cell death. In comparison, Ad5-GFP, which lacks the early gene promoter region (E1a) which is required for viral replication and expresses the GFP gene, induced 86% cell death when applied at 500 viral particles per cell, which translates to 219 pentons per cell (**Fig. 9**). This data suggests that human cells can better tolerate a high

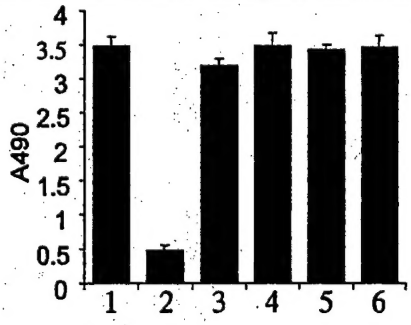


**Figure 9.** Testing the cytotoxicity of 3POF versus Ad5 on HeLa cells in culture. Substrate conversion by living cells results in a dark preceipitate whose concentration is measured by spectrophotometric absorbance at 490 nm (A490). Product formation is directly proportional to cell survival. 1, untreated cells; 2, Ad5-GFP; 3, Protamine + DNA; 4, 3PO; 5, F3PO; 6, Fiber alone.

concentration of pentons delivered through F3PO rather than Ad5, and that the cytotoxic effects of the virus are due to other additional factors.

*Task 3.* To test the ability of recombinant fiber-penton complexes to deliver the lacZ and HSV-tk genes to breast cancer cells in an animal model and determine the therapeutic efficacy of this novel gene delivery system *in vivo.*

Testing extent of immune response. Although a major goal of this research is to determine the efficacy of targeted gene delivery *in vivo*, it is also important to consider the immune effects of this gene delivery system. To determine the extent of immune response to the gene delivery system, complexes were assembled and injected into immune-competent mice (strain C57BL/6). To first determine the optimal assembly requirements for injections using minimal aqueous volumes, 3PO protein components, PBK10 and protamine, were both titrated against one another and the resulting complexes were tested for gene delivery of Luc using a high throughput luciferase assay (Steady-Glo, Promega Corporation, Madison, WI, USA). Using this assay, complexes could be formed using a DNA:PBK10:protamine weight ratio of 1:0.1:1.

Complexes were formed using the ratios just described and either injected intramuscularly or given orally in 50 microliter (ul) volumes per anesthetized mouse. Mice were injected on days 0, 7, 14, and 21. Orbital blood samples were collected from anesthetized animals on days 0, 7, 14, 21, and 35, and processed for ELISA analysis. End point titers for each serum sample are the reciprocal of the highest dilution that produces an average O.D. reading greater than 0.200.

Below is a table summarizing the parameters of the immune study:

| Group # | Antigen | Delivery Route | Dose DNA | Day of Injection | Blood collection |
|---|---|---|---|---|---|
| A | DNA | IM | 10μg | 0,7,14,21 | 0,7,14,35 |
| B | DNA + penton | IM | 10μg | 0,7,14,21 | 0,7,14,35 |
| C | DNA +penton + fiber | IM | 10μg | 0,7,14,21 | 0,7,14,35 |
| D | DNA | Oral | 10μg | 0,7,14,21 | 0,7,14,35 |
| E | DNA + penton | Oral | 10μg | 0,7,14,21 | 0,7,14,35 |
| F | DNA +penton + fiber | Oral | 10μg | 0,7,14,21 | 0,7,14,35 |
| G | Non-vaccinate controls | | None | None | 0,7,14,35 |

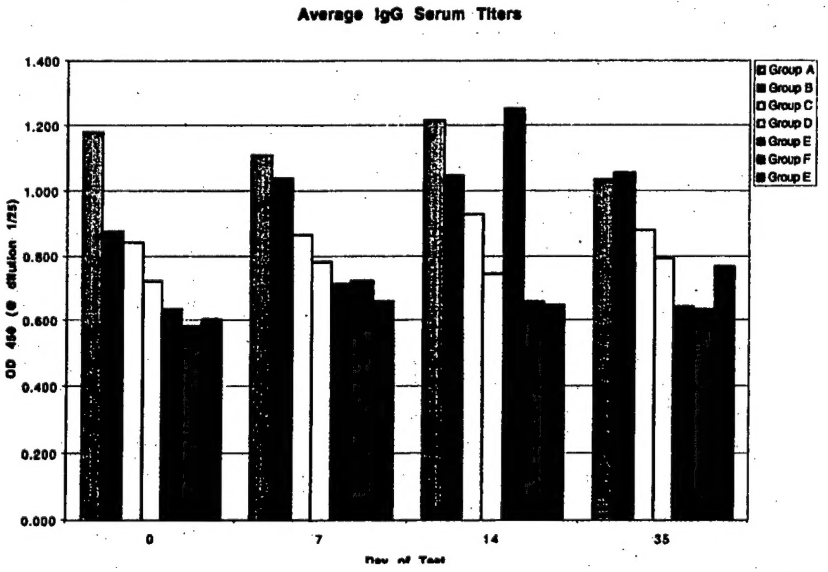


**Figure 10.** IgG serum titers after complex administration.

No significant difference was observed between animal groups injected with 3PO or 3PO + wild-type fiber, over the plasmid alone (**Fig. 10**). No significant difference was observed between groups of animals vaccinated by IM as compared to oral/nasal routes of administration.

Analyses on intracellular trafficking of recombinant capsid proteins. Although our heregulin-targeted proteins bound their target receptors at saturatable conditions, final transduction by targeted complexes *in vitro* remained low. We wanted to improve the transduction capacity of our vectors prior to *in vivo* testing, and therefore investigated the intracellular trafficking of the capsid proteins to determine which step during gene transfer required improvement.

We incubated either recombinant fiber or penton with HeLa cells at 4°C to promote receptor binding, then warmed the cells at 37°C to promote endocytosis of bound proteins. Rhodamine-phalloidin staining of actin filaments in WTF-treated cells shows reorganization of the actin cytoskeleton with filipodia-like extensions protruding from the cell peripheries after binding **(Fig. 11a)**, whereas penton (not shown) and BSA-treated control cells show no such alteration **(Fig. 11b)**. Surprisingly, immunostaining for fiber showed that the protein was taken up by cells and localized strongly to the nucleus at 37°C **(Fig. 11c)**. In contrast, penton immunostaining shows a distinct perinuclear accumulation at 37°C **(Fig. 11d)**. Interestingly, fiber internalization is also detected in cells treated at 4°C only, suggesting that uptake does not occur by classical receptor-mediated endocytosis but rather by a process that is temperature and thus energy independent **(Fig. 11e)**. This contrasts with penton, which is retained on the cell surface at 4°C **(Fig. 11f)**. We obtained images at 1 micron (μm) intervals along the Z-axis of the microscopic viewing plane to confirm that internalized fiber is located inside of the nucleus (not shown).

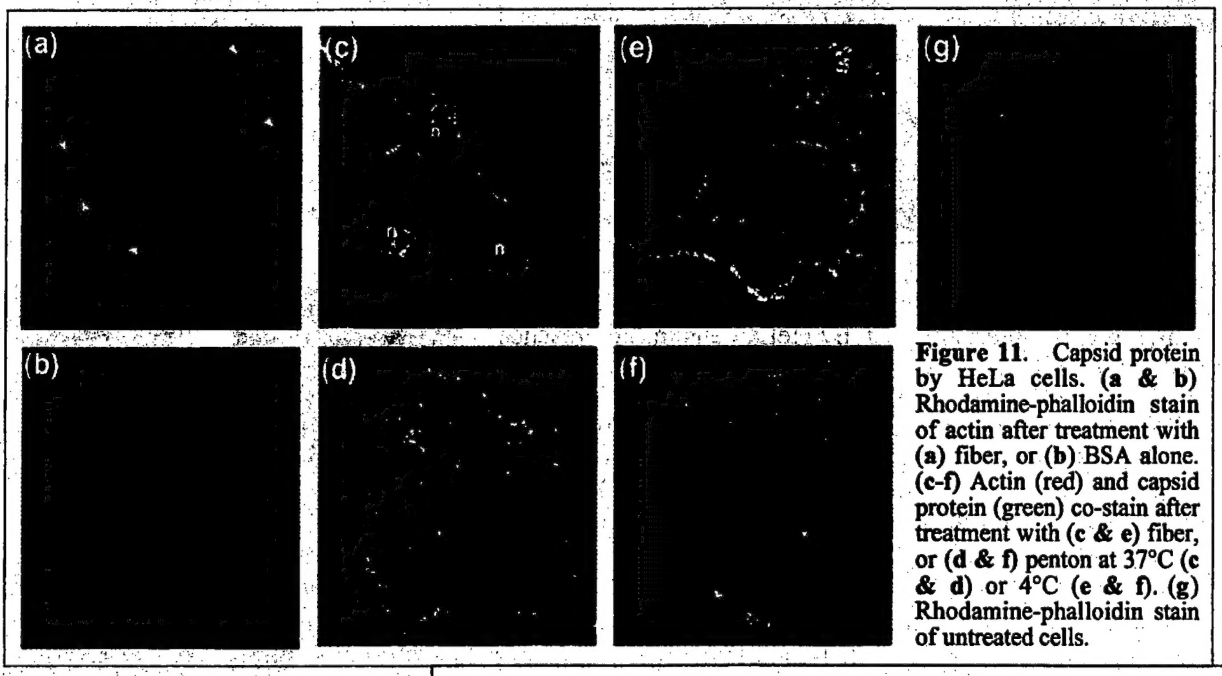


**Figure 11.** Capsid protein by HeLa cells. **(a & b)** Rhodamine-phalloidin stain of actin after treatment with **(a)** fiber, or **(b)** BSA alone. **(c-f)** Actin (red) and capsid protein (green) co-stain after treatment with **(c & e)** fiber, or **(d & f)** penton at 37°C **(c & d)** or 4°C **(e & f)**. **(g)** Rhodamine-phalloidin stain of untreated cells.

The fiber receptor, CAR, does not internalize. To determine whether fiber uptake is mediated by CAR internalization, we used an established assay that detects the internalization of soluble recombinant GFP-tagged ligands bound to their respective receptors (Medina-Kauwe et al., 2000; See **Appendix 2**). We have shown previously that a GFP-heregulin fusion protein (GFP-Her) binds specifically to the heregulin receptor, and that receptor mediated endocytosis in MDA-MB-453 cells can be detected microscopically and by FACS assay after trypsin/EDTA-removal of cell surface bound proteins. We found that up to 80% of cell surface bound GFP-Her is internalized by 30 minutes in

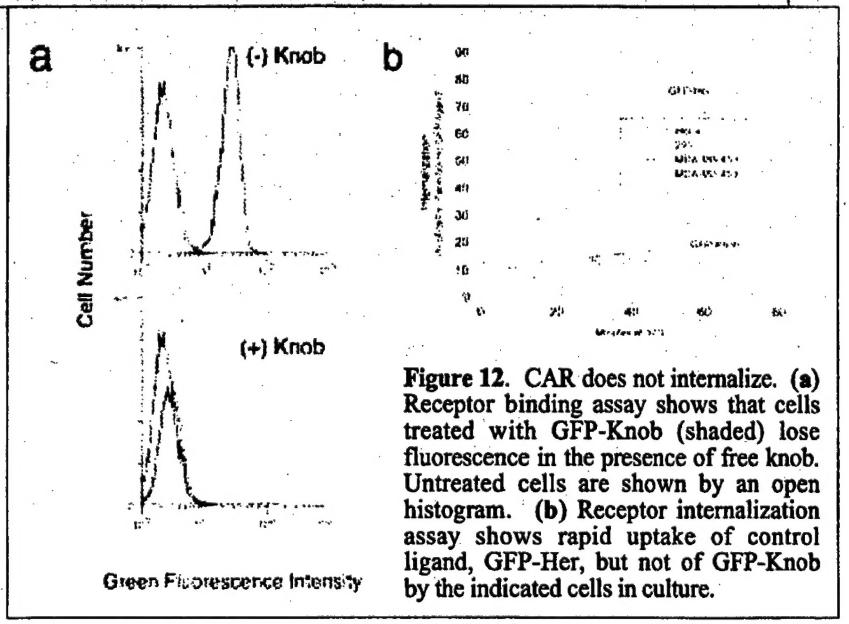


**Figure 12.** CAR does not internalize. **(a)** Receptor binding assay shows that cells treated with GFP-Knob (shaded) lose fluorescence in the presence of free knob. Untreated cells are shown by an open histogram. **(b)** Receptor internalization assay shows rapid uptake of control ligand, GFP-Her, but not of GFP-Knob by the indicated cells in culture.

MDA-MB-453 cells, which express both CAR and the heregulin receptor. We used the same assay to detect internalization of a GFP-Knob fusion protein. Specific receptor binding by GFP-Knob was confirmed through competitive inhibition by free knob on HeLa cells (**Fig. 12a**). Similar receptor binding to 293 and MDA-MB-453 cells is also reported (25, 27). However, even at up to 60 minutes at 37°C, little to no GFP-Knob internalization is detected in either HeLa, 293, or MDA-MB-453 cells (**Fig. 12b**).

The fiber tail/shaft induces cytoskeletal changes. To determine whether the cytoskeletal changes we observed following exposure of cells to the fiber is due to CAR binding or other factors, we incubated Hela cells with either full-length fiber or just the knob moiety in equivalent molar concentrations. After incubation with the fiber for 1 hour, cells developed branch-like extensions that stained positive for paxillin (**Fig. 13a**), identifying these extensions as focal adhesions. Although localized areas of the cell peripheries stained positive for paxillin in knob-treated cells, cytoskeletal alteration was minimal (**Fig. 13b**). Fewer to no filipodial extensions per cell were noticeable after knob treatment in comparison to fiber treatment, with the extensions themselves appearing shorter, broader, and less extended. These findings suggest that the fiber shaft rather than the knob is an important contributing factor in the cellular response to fiber binding. Of note, we also found that fiber-treated cells were more adherent to the coverslip than buffer-treated control cells (**Fig. 13c**), which became more loosely attached during incubation in buffer.

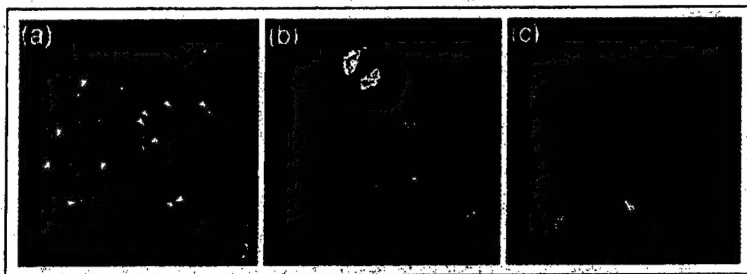


**Figure 13.** Immunofluorescence of HeLa treated with (**a**) fiber or (**b**) knob. Cells were stained with rhodamine-phalloidin (red) and immunostained for paxillin (green). Regions of co-localization appear yellow. Arrowheads point to filipodial extensions containing localized concentrations of paxillin stain. (**c**) Untreated cells. Cell numbers in (**b**) and (**c**) are reduced compared to (**a**) due to cell lifting in (**b**) and (**c**).

Penton uptake requires an intact cytoskeleton. To determine the cellular requirements for penton uptake and trafficking, we investigated penton internalization by HeLa cells in the presence of either: cytochalasin D or nocodazole, inhibitors of actin filament or microtubule polymerization, respectively. Our findings show that cytochalasin D inhibits internalization (**Fig. 14, "+ Cyto D"**) whereas nocodazole prevents perinuclear localization but not internalization (**Fig. 14, "+ Noc"**). These findings suggest that intact actin filaments are required for endocytosis whereas intact microtubules are required for intracellular trafficking toward the nucleus but not endocytosis.

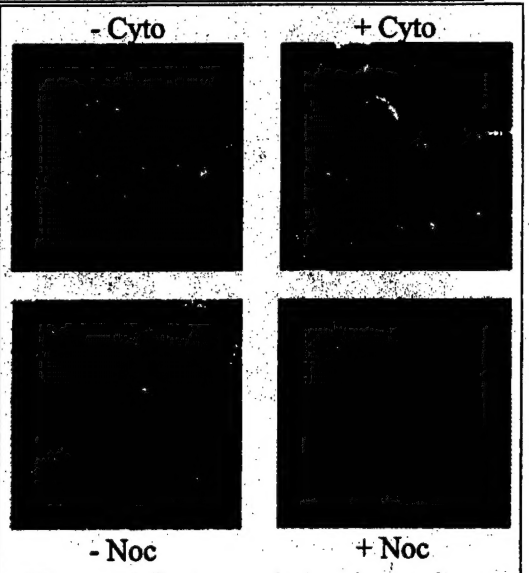


**Figure 14.** Penton uptake requires an intact cytoskeleton.

These trafficking studies begin to address some issues regarding the molecular requirements for cell entry of recombinant capsid proteins. We find here that the penton requires an endocytic pathway governed by actin-mediated internalization and microtubule-mediated intracellular trafficking. Perinuclear accumulation of the penton protein after uptake suggests that delivery of exogenous DNA to the nucleus may require trafficking by protamine after cell entry of complexes or by cell division after cell entry. The nuclear localization of the fiber protein, on the other hand, suggests an additional importance of the fiber not just for cell binding but also for intracellular trafficking. Moreover, that the shaft dictates translocation of the fiber across cell membranes predicts an encouraging outcome when targeted fibers are combined with our penton-DNA complexes. We have actually found that the fiber protein alone can mediate gene delivery to HeLa and 293 cells, albeit at an 8% transduction efficiency. The fiber directs 3PO transduction at an equal level of transduction efficiency. Thus, whereas the fiber can enhance uptake of complexes, gene transfer remains low. The efficient trafficking of the capsid proteins to distinct subcellular compartments after cell entry, however, suggest that intracellular translocation of the capsid proteins is not a limiting factor to gene transfer, and, therefore, improvements should be made to other aspects of the gene delivery complexes, such as complex size.

## KEY RESEARCH ACCOMPLISHMENTS

Production and characterization of recombinant penton protein (PBK10)

Assembly of penton-based gene delivery complexes (3PO)

Demonstration of non-viral gene delivery by 3PO

Production and characterization of targeted penton protein (HerPBK10)

Demonstration of heregulin-targeted gene delivery by recombinant targeted penton complexes (H2PO)

Production and characterization of wild-type (WTF) and targeted fiber protein (FVL-Her)

Assembly of fiber-penton gene delivery complexes (3POF)

Demonstration of fiber-directed gene delivery by 3POF gene delivery complexes

Determination of cytotoxicity of capsid proteins compared to whole virus

Demonstration of fiber and penton intracellular trafficking

## REPORTABLE OUTCOMES

Manuscripts

L.K. Medina-Kauwe, V. Leung, L. Wu, and L. Kedes (2000) Assessing the binding and endocytosis activity of cellular receptors using GFP-ligand fusions. *BioTechniques* 29:602-609.

L.K. Medina-Kauwe, N. Kasahara, and L. Kedes (2000) 3PO, a novel non-viral gene delivery system using engineered Ad5 penton proteins. *Gene Therapy* 8, 795-803.

L.K. Medina-Kauwe, N. Kasahara, and L. Kedes (2001) Non-viral gene delivery to human breast cancer cells by targeted Ad5 penton proteins. *Gene Therapy* 8, 1753-1761.

Medina-Kauwe, LK., and Chen, X. (2002) Using GFP-Ligand Fusions to Measure Receptor-Mediated Endocytosis in Living Cells. *In* "Vitamins and Hormones" (G Litwack, Ed), Vol. 65, pp. 81-96. Academic Press, San Diego.

L.K. Medina-Kauwe, X. Chen, M. Maguire, M. MacVeigh, S. Hamm-Alvarez and L. Kedes (2002) The Ad5 fiber translocates through cell membranes and localizes to the nucleus, in preparation.

Abstracts

L.K. Medina-Kauwe, N. Kasahara, and L. Kedes (2000) Nonviral gene delivery to human breast cancer cells by targeted Ad5 penton proteins, Molecular Therapy Abstracts 1(5), 292.

L.K. Medina-Kauwe, Meghan Maguire, John Daoud, Elizabeth Felix-Trunnelle, Noriyuki Kasahara, Larry Kedes (2001) Non-Viral Gene Delivery by Targeted Adenovirus Capsid Proteins, Molecular Therapy 3 (5), 33.

L.K. Medina-Kauwe, M. Maguire, N. Kasahara, and L. Kedes (2002) A novel system for targeting gene delivery to breast cancer cells. Era of Hope 2002, DoD Breast Cancer Research Program Meeting Abstracts.

Presentations

2000 "Genetic Engineering of Viral Proteins to Provide Targeted Non-Viral Gene Therapy to Breast Cancer Cells", Susan G. Komen Breast Cancer Foundation Fourth Annual Conference, September 16-19, Washington D.C.

2000 "Every Time It Rains, It Rains Pentons From Heaven", USC Department of Biochemistry Retreat, November 12-14, Desert Hot Springs, California.

2001 "Fashionable Clones and Designer Genes", Science Teaching Conference, Dorris Place Elementary School, January 22, Los Angeles, California.

2001 "Engineering Adenovirus Capsid Proteins for Non-Viral Gene Delivery", Selective Genetics Inc., March 12, San Diego, California.

2001 "Ad5 Capsid Protein Trafficking and Non-Viral Gene Therapy", UCLA Department of Medicine Gene Medicine Monday Forum, October 22, Los Angeles, California.

2001 "Intracellular Trafficking and Non-Viral Gene Delivery by Ad5 Capsid Proteins", USC Department of Pharmaceutical Sciences Seminars, November 16, Los Angeles, California.

2002"Ad5 Capsid Protein Trafficking in Hepatocytes: Pathology and Therapy", USC Liver Center Symposium, February 16, Los Angeles, California.

2002 "Ad5 Capsid Protein Uptake and Trafficking in Epithelial Cells", West Coast Salt & Water Club Annual Meeting, March 8-9, Morro Bay, California.

Employment/Research opportunities received

Appointment of Dr. Lali K. Medina-Kauwe to Assistant Professor of Research in the University of Southern California Department of Biochemistry.

## CONCLUSIONS

The concept of non-viral gene delivery by Ad5 capsid proteins has been established with the development of 3PO. We have shown that the Ad5 penton can be engineered to contain a DNA binding sequence, and that this recombinant protein (PBK10) can mediate gene delivery to cells in culture. We have also observed that the fiber protein enhances penton-mediated gene delivery, and specifically targets delivery to the fiber receptor, CAR. It has now been shown that a targeted fiber protein (FVL-Her) can be made and retains the trimerization of the wild-type fiber by the inclusion of a viral trimerization element. These receombinant proteins are non-toxic to cells and did not elicit a remarkable immune response in mice. The proteins bind efficiently to their respective receptors, however gene transfer was lower than desired. To determine where improvements can be made in gene transfer efficiency, we investigated the intracellular trafficking of the capsid proteins after cell binding and entry. These studies on capsid protein trafficking are the first to be performed to our knowledge. We found that the fiber protein can be internalized in the absence of the penton. We also found that fiber and penton proteins translocate to distinct subcellular compartments: the fiber localizes to the nucleus whereas the penton accumulates around the perinuclear envelope. Penton uptake is temperature dependent whereas fiber uptake is not. These studies on intracellular trafficking of the capsid proteins are being submitted for publication. Our future studies will include investigating the trafficking of the whole complexes in comparison with the individual capsid proteins. We will also investigate whether physical parameters such as complex size regulate gene transfer.

## REFERENCES

Bacus, S. S., Zelnick, C. R., Plowman, G., and Yarden, Y. (1994). Expression of the erbB-2 family of growth factor receptors and their ligands in breast cancers. Implication for tumor biology and clinical behavior. American Journal of Clinical Pathology 102, S13-24.

Bergelson, J. M., Cunningham, J. A., Droguett, G., Kurt-Jones, E. A., Krithivas, A., Hong, J. S., Horwitz, M. S., Crowell, R. L., and Finberg, R. W. (1997). Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. Science 275, 1320-3.

Carraway, K. L., 3rd, and Cantley, L. C. (1994). A neu acquaintance for erbB3 and erbB4: a role for receptor heterodimerization in growth signaling. Cell 78, 5-8.

Carraway, K. L., 3rd, Sliwkowski, M. X., Akita, R., Platko, J. V., Guy, P. M., Nuijens, A., Diamonti, A. J., Vandlen, R. L., Cantley, L. C., and Cerione, R. A. (1994). The erbB3 gene product is a receptor for heregulin. Journal of Biological Chemistry 269, 14303-6.

Carraway, K. L., 3rd, and Burden, S. J. (1995). Neuregulins and their receptors. Current Opinion in Neurobiology 5, 606-12.

Culver, K. W., Vickers, T. M., Lamsam, J. L., Walling, H. W., and Seregina, T. (1995). Gene therapy for solid tumors. British Medical Bulletin 51, 192-204.

Fisher, K. J., and Wilson, J. M. (1997). The transmembrane domain of diphtheria toxin improves molecular conjugate gene transfer. Biochemical Journal 321, 49-58.

Fominaya, J., and Wels, W. (1996). Target cell-specific DNA transfer mediated by a chimeric multidomain protein. Novel non-viral gene delivery system. Journal of Biological Chemistry 271, 10560-8.

Fominaya, J., Uherek, C., and Wels, W. (1998). A chimeric fusion protein containing transforming growth factor-a mediates gene transfer via binding to the EGF receptor. Gene Therapy 5, 521-30.

Gao, X., and Huang, L. (1996). Potentiation of cationic liposome-mediated gene delivery by polycations. Biochemistry 35, 1027-36.

Goldman, R., Levy, R. B., Peles, E., and Yarden, Y. (1990). Heterodimerization of the erbB-1 and erbB-2 receptors in human breast carcinoma cells: a mechanism for receptor transregulation. Biochemistry 29, 11024-8.

Gottschalk, S., Sparrow, J. T., Hauer, J., Mims, M. P., Leland, F. E., Woo, S. L., and Smith, L. C. (1996). A novel DNA-peptide complex for efficient gene transfer and expression in mammalian cells. Gene Therapy 3, 48-57.

Greber, U. F., Willetts, M., Webster, P., and Helenius, A. (1993). Stepwise dismantling of adenovirus 2 during entry into cells. Cell 75, 477-86.

Han, X., Kasahara, N., and Kan, Y. W. (1995). Ligand-directed retroviral targeting of human breast cancer cells. Proceedings of the National Academy of Sciences of the United States of America 92, 9747-51.

Harris, C. E., Agarwal, S., Hu, P., Wagner, E., and Curiel, D. T. (1993). Receptor-mediated gene transfer to airway epithelial cells in primary culture. American Journal of Respiratory Cell & Molecular Biology 9, 441-7.

Holmes, W. E., Sliwkowski, M. X., Akita, R. W., Henzel, W. J., Lee, J., Park, J. W., Yansura, D., Abadi, N., Raab, H., Lewis, G. D., and et al. (1992). Identification of heregulin, a specific activator of p185erbB2. Science 256, 1205-10.

Hung, M. C., Matin, A., Zhang, Y., Xing, X., Sorgi, F., Huang, L., and Yu, D. (1995). HER-2/neu-targeting gene therapy--a review. Gene 159, 65-71.

Jeschke, M., Wels, W., Dengler, W., Imber, R., Stocklin, E., and Groner, B. (1995). Targeted inhibition of tumor-cell growth by recombinant heregulin-toxin fusion proteins. International Journal of Cancer 60, 730-9.

Karayan, L., Hong, S. S., Gay, B., Tournier, J., d'Angeac, A. D., and Boulanger, P. (1997). Structural and functional determinants in adenovirus type 2 penton base recombinant protein. Journal of Virology 71, 8678-89.

Kozarsky, K. F., and Wilson, J. M. (1993). Gene therapy: adenovirus vectors. Current Opinion in Genetics & Development 3, 499-503.

Lenferink, A. E., Pinkas-Kramarski, R., van de Poll, M. L., van Vugt, M. J., Klapper, L. N., Tzahar, E., Waterman, H., Sela, M., van Zoelen, E. J., and Yarden, Y. (1998). Differential endocytic routing of homo- and hetero-dimeric ErbB tyrosine kinases confers signaling superiority to receptor heterodimers. EMBO Journal 17, 3385-97.

Li, W., Park, J. W., Nuijens, A., Sliwkowski, M. X., and Keller, G. A. (1996). Heregulin is rapidly translocated to the nucleus and its transport is correlated with c-myc induction in breast cancer cells. Oncogene 12, 2473-7.

Medina-Kauwe, L. K., Leung, V., Wu, L., and Kedes, L. (2000). Assessing the Binding and Endocytosis Activity of Cellular Receptors Using GFP-Ligand Fusions. BioTechniques 29, 602-609.

Medina-Kauwe, L. K., Kasahara, N., and Kedes, L. (2001a). 3PO, a novel non-viral gene delivery system using engineered Ad5 penton proteins. Gene Therapy 8, 795-803.

L.K. Medina-Kauwe, N. Kasahara, and L. Kedes (2001) Non-viral gene delivery to human breast cancer cells by targeted Ad5 penton proteins. *Gene Therapy*, 8:1753-1761.

Moorman, D. W., and Culver, K. W. (1995). Gene transfer and cancer chemotherapy. Molecular & Cell Biology of Human Diseases Series 5, 125-139.

O'Keefe, D. O., Cabiaux, V., Choe, S., Eisenberg, D., and Collier, R. J. (1992). pH-dependent insertion of proteins into membranes: B-chain mutation of diphtheria toxin that inhibits membrane translocation, Glu-349---Lys. Proceedings of the National Academy of Sciences of the United States of America 89, 6202-6.

Prchla, E., Plank, C., Wagner, E., Blaas, D., and Fuchs, R. (1995). Virus-mediated release of endosomal content in vitro: different behavior of adenovirus and rhinovirus serotype 2. Journal of Cell Biology 131, 111-23.

Press, M. F., Jones, L. A., Godolphin, W., Edwards, C. L., and Slamon, D. J. (1990). HER-2/neu oncogene amplification and expression in breast and ovarian cancers. Progress in Clinical & Biological Research 354A, 209-21.

Seth, P., Fitzgerald, D., Ginsberg, H., Willingham, M., and Pastan, I. (1984). Evidence that the penton base of adenovirus is involved in potentiation of toxicity of Pseudomonas exotoxin conjugated to epidermal growth factor. Molecular & Cellular Biology 4, 1528-33.

Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A., and McGuire, W. L. (1987). Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 235, 177-82.

Slamon, D. J., and Clark, G. M. (1988). Amplification of c-erbB-2 and aggressive human breast tumors? Science 240, 1795-8.

Sliwkowski, M. X., Schaefer, G., Akita, R. W., Lofgren, J. A., Fitzpatrick, V. D., Nuijens, A., Fendly, B. M., Cerione, R. A., Vandlen, R. L., and Carraway, K. L., 3rd (1994). Coexpression of erbB2 and erbB3 proteins reconstitutes a high affinity receptor for heregulin. Journal of Biological Chemistry 269, 14661-5.

Tzahar, E., Levkowitz, G., Karunagaran, D., Yi, L., Peles, E., Lavi, S., Chang, D., Liu, N., Yayon, A., Wen, D., and et al. (1994). ErbB-3 and ErbB-4 function as the respective low and high affinity receptors of all Neu differentiation factor/heregulin isoforms. Journal of Biological Chemistry 269, 25226-33.

Uherek, C., Fominaya, J., and Wels, W. (1998). A modular DNA carrier protein based on the structure of diphtheria toxin mediates target cell-specific gene delivery. Journal of Biological Chemistry 273, 8835-41.

Waterman, H., Sabanai, I., Geiger, B., and Yarden, Y. (1998). Alternative intracellular routing of ErbB receptors may determine signaling potency. Journal of Biological Chemistry 273, 13819-27.

Wickham, T. J., Mathias, P., Cheresh, D. A., and Nemerow, G. R. (1993). Integrins avb3 and avb5 promote adenovirus internalization but not virus attachment. Cell 73, 309-19.

Wickham, T. J., Filardo, E. J., Cheresh, D. A., and Nemerow, G. R. (1994). Integrin alpha v beta 5 selectively promotes adenovirus mediated cell membrane permeabilization. Journal of Cell Biology 127, 257-64.

Yang, Y., Ertl, H. C., and Wilson, J. M. (1994). MHC class I-restricted cytotoxic T lymphocytes to viral antigens destroy hepatocytes in mice infected with E1-deleted recombinant adenoviruses. Immunity 1, 433-42.

Yarden, Y., and Weinberg, R. A. (1989). Experimental approaches to hypothetical hormones: detection of a candidate ligand of the neu protooncogene. Proceedings of the National Academy of Sciences of the United States of America 86, 3179-83.

## APPENDIX

A.1 – Publication reprint

A.2 - Publication reprint

A.3 - Publication reprint

Gene Therapy (2001) 8, 795–803

www.nature.com/gt

RESEARCH ARTICLE

# 3PO, a novel nonviral gene delivery system using engineered Ad5 penton proteins

LK Medina-Kauwe[1], N Kasahara[1,2] and L Kedes[1]

*Institute for Genetic Medicine and Departments of [1]Biochemistry and Molecular Biology, and [2]Pathology, University of Southern California Keck School of Medicine, Los Angeles, CA, USA*

*This study describes the development of 3PO, a nonviral, protein-based gene delivery vector which utilizes the highly evolved cell-binding, cell-entry and intracellular transport functions of the adenovirus serotype 5 (Ad5) capsid penton protein. A penton fusion protein containing a polylysine sequence was produced by recombinant methods and tested for gene delivery capability. As the protein itself is known to bind integrins through a conserved consensus motif, the penton inherently possesses the ability to bind and enter cells through receptor-mediated internalization. The ability to lyse the cellular endosome encapsulating internalized receptors is also attributed to the penton. The recombinant protein gains the additional function of DNA binding and transport with the appendage of a polylysine motif. This protein retains the ability to form pentamers and mediates delivery of a reporter gene to cultured cells. Interference by oligopeptides bearing the integrin binding motif suggests that delivery is mediated specifically through integrin receptor binding and internalization. The addition of protamine to penton–DNA complexes allows gene delivery in the presence of serum.* Gene Therapy (2001) **8**, 795–803.

**Keywords:** *3PO; PBK10; penton; protamine; adenovirus; gene therapy*

## Introduction

Nonviral methods of gene delivery have remained an attractive alternative to virus-based gene therapy, mainly because of their safety and low toxicity. Their historically low efficiency of gene transfer and expression, particularly *in vivo*, requires the development of novel strategies to improve delivery. Directing nonviral vectors to cellular receptors by the incorporation of specific ligands, such as transferrin, asialoglycoprotein, or epithelial growth factor (EGF), has provided an efficient means of crossing the plasma membrane by receptor-mediated internalization.[1–13] The cellular endosome encapsulating the vector following endocytosis, however, has proven to be a major barrier to nuclear delivery and gene expression by nonviral vectors.[14] Without endosome penetration, lysosomal enzymes eventually degrade the DNA, resulting in low transduction.[15,16] As viruses have evolved the means to penetrate these barriers, the inclusion of viral fusogenic peptides or endosomolytic agents into nonviral vectors has enhanced the gene delivery capacity of nonviral systems.[12,17,18] Attention has been focused on adenovirus capsid proteins for this purpose. It has been shown that the addition of whole, inactivated adenovirus to plasmid conjugates significantly enhances plasmid gene delivery, implying that the adenovirus capsid provides a means for endosomal penetration.[19–22] As the next logical step we have attempted to capture this function using only the proteins necessary for imparting cellular uptake and penetration.

The highly efficient cell binding and entry functions imparted by the capsid proteins of adenovirus serotype 5 (Ad5) are a major reason why adenoviruses have been widely used as vehicles for gene transfer.[23,24] The adenovirus capsid is an icosahedral structure with long antenna-like protrusions at each of its 12 vertices.[25] Each protrusion consists of a homotrimeric fiber protein, at the base of which lies a homopentameric penton base.[26,27] The first step of infection by Ad5 involves high affinity binding of the fiber homotrimer to the Coxsackievirus adenovirus receptor (CAR), a ubiquitous component of mammalian cellular membranes.[28] This is followed by secondary binding of the penton base to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins through an Arg-Gly-Asp (RGD) motif located in the middle of the penton protein sequence.[29–31] Binding of the penton to $\alpha_v$ integrins triggers receptor-mediated internalization of the whole virus and subsequent encapsulation into endosomes.[32,33] Vesicle penetration occurs in response to endosome acidification and appears to be mediated by the penton, thus allowing viral access to the cytoplasm.[34–36] It has been shown that whereas the fiber receptor fails to internalize in response to ligand binding, $\alpha_v$ integrins internalize rapidly after binding to penton proteins.[37] Thus, whereas the binding event that initiates infection by Ad5 is dictated by the fiber, this event is independent of and separable from the secondary binding and subsequent internalization by the penton.

A second integrin binding motif, LDV, is also found in the penton peptide sequence. This core sequence mediates binding to $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins, which are

*Correspondence: L Kedes, Institute for Genetic Medicine and Department of Biochemistry and Molecular Biology, University of Southern California Keck School of Medicine, 2250 Alcazar Street CSC240, Los Angeles, California 90033, USA*
Received 16 July 2000; 31 January 2001

predominantly found on lymphocytes, monocytes, and fibroblasts.[38,39] It has been shown that mutation of this site affects binding to HeLa cells as well, implying that HeLa cells express both $\alpha_v$ and $\alpha_4$ integrins.[36]

To explore the use of engineered Ad5 pentons for protein-based gene delivery, soluble proteins were produced by recombinant DNA methods. It has been shown that soluble wild-type penton proteins retain the features of pentamer formation, receptor binding and endocytosis, and assembly with fiber proteins.[27,37,40,41] In order to take advantage of these characteristics for nonviral gene delivery, we have engineered the Ad5 penton protein to bind and transport DNA in the absence of whole virus. In the process of developing this protein for gene therapy, we have tested its ability to mediate gene delivery to cells in culture. Here, we report our *in vitro* findings.

## Results

### *Characterization of recombinant penton proteins*

Previous studies have shown that the appendage of eight to 10 cationic residues to synthetic peptides is adequate for DNA binding.[17] Therefore, we introduced a decalysine sequence at the carboxy (C) terminus of the Ad5 penton protein by genetic modification (Figure 1a), and recombinant proteins were produced in *E. coli*. As the lysine tag comprises a short stretch of residues, the predicted molecular size of the modified protein is increased only slightly compared with the unmodified protein. As expected, both lysine-tagged (PBK10) and untagged (PB) penton proteins purified from bacterial lysates similarly migrate near 68 kDa on single percentage gels (Figure 1b, left). On a gradient gel, which better distinguishes the sizes of the two proteins, the lysine-tagged penton exhibits a slightly slower migration rate (Figure 1b, right), presumably due to both its additional cationic charge and slightly higher molecular weight. Polyclonal antiserum specific to Ad5 capsid proteins recognizes both the lysine-tagged and untagged penton proteins, thus confirming their identities (Figure 1b, right).

Previous studies using nondenaturing PAGE analysis demonstrated that both viral and recombinant wild-type soluble penton proteins form homopentamers in the absence of additional viral or other scaffolding proteins.[41] We used nondenaturing PAGE to determine the oligomer size formed by PBK10. Under these conditions, PB migrates according to its expected size of approximately 335 kDa (Figure 1c). PBK10 migrates slower, which is in agreement with homopentamer formation at the predicted size of 345 kDa and its higher cationic charge (Figure 1c). These results indicate that the additional charged residues at the penton C-terminus do not impede pentamer assembly.

It has been well established that the Ad5 penton binds to $\alpha_v$ integrins, which are present on numerous epithelial cell lines, including 293 and HeLa cells.[39] We have previously demonstrated that GFP-tagged proteins can be used to detect receptor–ligand interactions by fluorescence activated cell sorting (FACS).[42] Accordingly, we constructed penton proteins tagged with green fluorescent protein (GFP) at their N-terminus. These proteins were also produced in bacteria and characterized by immunoblotting to confirm their structure (data not shown). We have shown previously that GFP does not interfere with wild-type oligomer formation of ligands.[42] The size of GFP-tagged penton oligomers is difficult to determine, however, as the final product size is too large to assess by standard nondenaturing PAGE analysis. To determine cell binding ability, the GFP-tagged penton proteins were incubated with 293 cells and analyzed by FACS scan. Increasing concentrations of recombinant proteins result in an increased fluorescence emission from treated cells, suggesting dose-dependent cell binding of recombinant proteins (Figure 1d). Importantly, a similar degree of mean fluorescent shift results from equivalent concentrations of lysine-tagged and untagged proteins, suggesting that the lysine sequence does not interfere with cell binding activity.

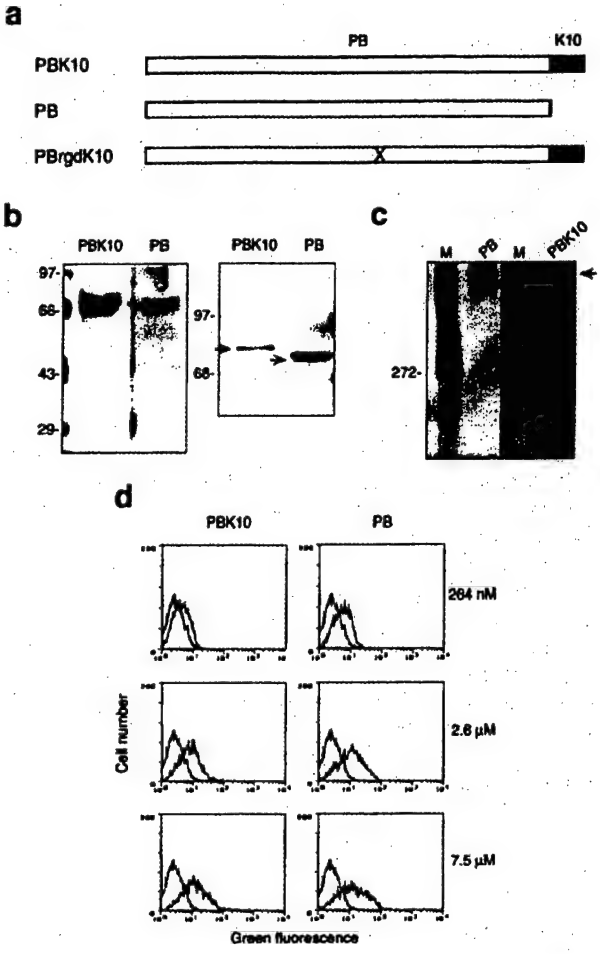


***Figure 1*** *Characterization of recombinant penton proteins. (a) Schematic of recombinant penton constructs (not drawn to scale). Each protein is represented by a linear bar. The decalysine sequence is depicted at the right (carboxy-terminal) end of each construct by a shaded box. The R340E mutation is depicted by an 'X'. (b) SDS-PAGE analysis of affinity-purified proteins. Left, Coomassie stain of purified proteins run on 7% acrylamide gels. Right, immunoblot of proteins run on a 4–12% gradient gel. Antibody used recognizes Ad5 capsid proteins. (c) Nondenaturing PAGE analysis of purified proteins. M, urease marker protein. (d) Cell binding analysis of recombinant penton proteins. FACScan results are overlaid to show fluorescence shift enhancement of 293 cells treated with increasing concentrations of each protein. White, untreated cells; Gray, treated cells.*

### *PBK10 binds DNA*

DNA binding ability can be determined by observing whether bound proteins slow DNA mobility on a gel

matrix. To test this, two such assays were performed. In the first assay, an oligonucleotide probe (81 ng) incubated with recombinant proteins exhibits a significant retardation in mobility in the presence of PBK10, but not with similar concentrations of PB or bovine serum albumin (BSA), indicating that DNA binding occurs specifically through the polylysine moiety (Figure 2a). The predicted amount of PBK10 to bind all of the probe is 1.75 μg, according to the polylysine:DNA phosphate charge ratio. Therefore, 1 μg of PBK10 is expected to bind approximately 60% of the probe. Densitometry scanning of the autoradiogram determines that about 70% of the probe is bound by PBK10, which is in close agreement with the predicted requirements. In the second assay, increasing concentrations of PBK10 incubated with constant amounts of plasmid DNA (pGFPemd-cmv) results in a concomitant DNA mobility reduction on an agarose gel (Figure 2b). Complete DNA charge neutralization and immobilization of the plasmid DNA would require a PBK10 to DNA (w/w) ratio of 22. In agreement with the predicted ratio, immobilization of the plasmid is achieved at a protein to DNA (w/w) ratio of between 11 and 23. Importantly, the results of both gel shift analyses show that PBK10 can bind both double-stranded circular forms, as well as single-stranded linear DNA.

### *PBK10 mediates gene delivery into cultured cells*

We then tested the ability of PBK10, without the GFP tag, to mediate delivery of plasmid DNA to cells in culture. Both 293 cells and HeLa cells express $\alpha_v$ integrins, and are commonly used for adenovirus infection procedures. Incubation of PBK10 with a reporter plasmid encoding GFP (pGFPemd-cmv) before adding the mixture to cells in culture produces detectable green fluorescence in 293 cells (Figure 3d) and in HeLa cells (Figure 3h), indicating both delivery and expression of the GFP gene. To determine the role of the polylysine tag in PBK10-mediated gene delivery, we compared PB with PBK10 by incubating them with pGFPemd-cmv at a protein to DNA ratio of 2 (w/w), and then adding them to monolayers of 293 cells. Under direct observation by UV light microscopy, PBK10 produces approximately three- to five-fold more fluorescing cells (Figure 3d) than PB (Figure 3c), or DNA alone (Figure 3b). Equivalent numbers of fluorescent cells are detected in wells treated with either PB + DNA or DNA alone, indicating that PB does not improve GFP expression without the lysine moiety. This suggests that DNA internalization occurs by direct association and transport with the lysine-tagged pentons, and is not an artifact of passive entry with the penton proteins.

To better measure gene delivery, 293 cells treated with penton–DNA complexes were analyzed by FACS. The complexes were formed at ratios selected for their likelihood to neutralize charged polylysine and DNA phosphate (w/w = 22). Cells were exposed to varying molar concentrations of complexes (3, 5, and 15 μM PBK10) in serum-free media. At all three PBK10 concentrations, the entire cell population shifts to a higher green fluorescence emission over background (Figure 4a and Table 1), indicating delivery to essentially every cell. Cells treated with 3 and 5 μM PBK10 suffer no visible toxicity. Although 15 μM PBK10 produced notable toxicity, seen as >50% necrotic cells and cellular debris, the remaining live cells emit visible green fluorescence (summarized in Table 1).

### *Deleterious effect of serum on gene delivery*

When gene delivery experiments identical to those just described were carried out in the presence of 1% serum, GFP expression is severely reduced and higher concen-

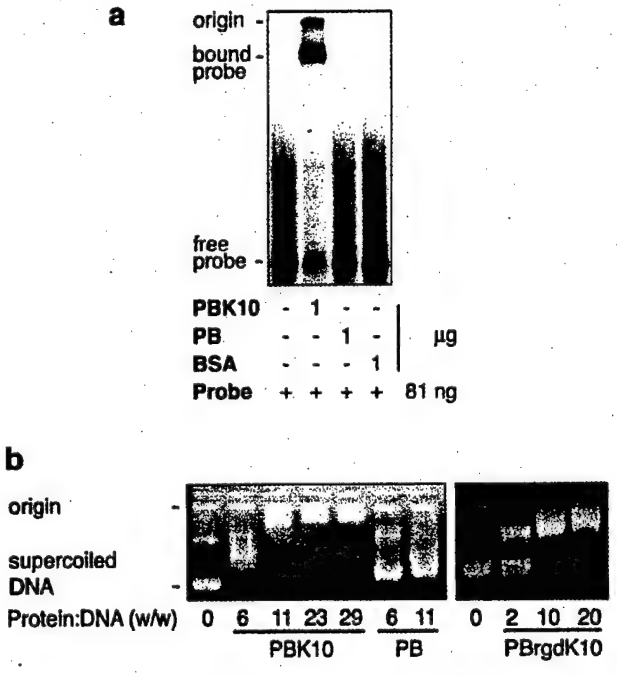


*Figure 2 PBK10 binds DNA. Mobility shift analyses of (a) an oligonucleotide probe, and (b) a 5 kb plasmid. Assays are performed as described in Materials and methods.*

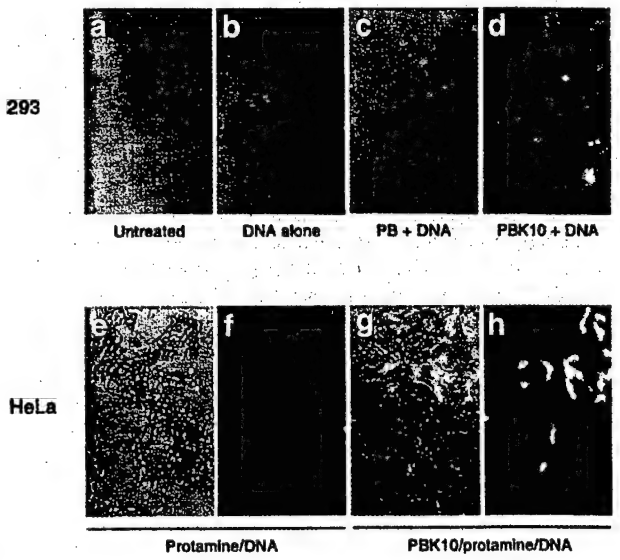


*Figure 3 PBK10 mediates gene delivery and GFP expression in cultured cells. (a–d) 293 cells at 10× magnification. DNA alone (5 μg) or mixed with penton proteins (10 μg) are exposed to cells as described in Materials and Methods. Cells are visualized by an FITC filter-polarized UV light. (e–h) HeLa cells at 10× magnification. DNA is mixed with protamine alone (7:1 w/w ratio of protamine:DNA) or with PBK10 (2:7:1 w/w ratio of PBK10:protamine:DNA) and exposed to cells as described. e and g, regular light; f and h, UV light.*

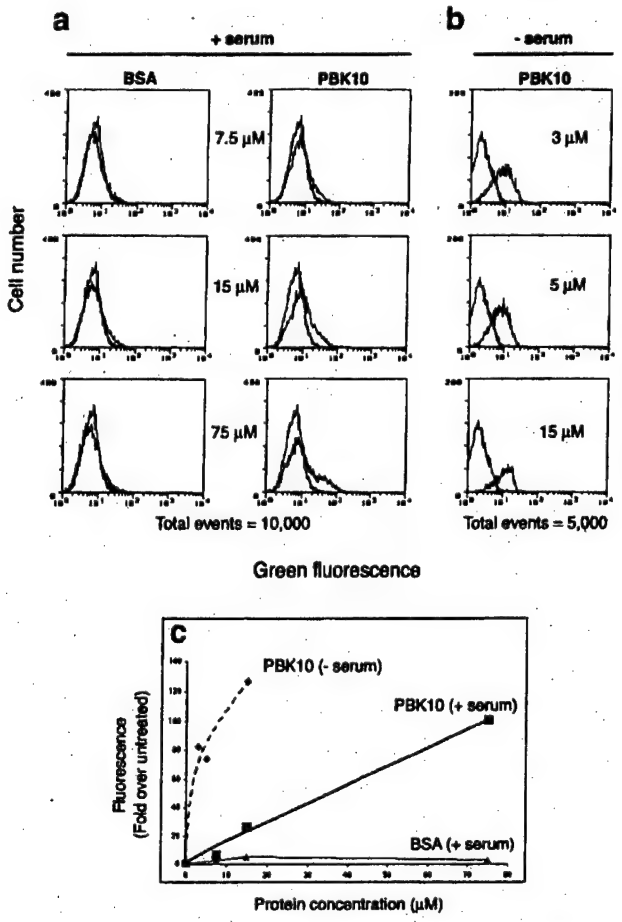


*Figure 4 FACS analysis of penton-mediated gene delivery and expression in 293 cells. (a and b) FACScans of treated cells. The untreated cell populations are shown in white. The treated cell populations are shown in gray. In (b), complexes are exposed to cells in the presence of 1% fetal bovine serum. (c) Summary of FACS data. Data points plot the number of gated fluorescent events of treated over untreated cells at each protein concentration assayed in (a) and (b).*

trations of PBK10 are required to detect any fluorescence over background. In order to test higher concentrations of PBK10, we first eliminated any free penton oligomers smaller than a pentamer that might compete with PBK10 + DNA complexes for cell binding. Accordingly, 7.5, 15, and 75 μM PBK10 or BSA were incubated with constant amounts of DNA before loading the complexes on to ultrafiltration columns that retain molecules greater than 300–900 K molecular weight. Cells were treated in the presence of 1% serum and green fluorescence was measured by FACS 3 days later. At all three doses, there is no detectable fluorescence from BSA-treated cells, whereas PBK10 appears to mediate DNA delivery dose-dependently and produces a significant number of GFP-positive cells (Figure 4b and Table 1). At 75 μM doses, the visible green fluorescence of PBK10-treated cells is 112-fold over background. In addition, little cytopathic effect is detectable, even at PBK10 concentrations as high as 75 μM, suggesting that serum has a protective effect against possible protein-induced cytotoxicity (summarized in Table 1). However, at comparative (15 μM) concentrations of PBK10, GFP expression is reduced by approximately 80% in the presence of serum. Figure 4c summarizes these FACS results and shows that the presence of 1% serum severely reduces gene delivery and expression.

### *Protamine protects against serum effects*

Several factors may produce the extreme reduction in PBK10-mediated gene delivery that is observed in the presence of serum. One of these may be the access of serum nucleases to the DNA, suggesting that PBK10 is unable to fully protect the plasmid DNA from nuclease-induced damage. The addition of small cationic molecules, such as protamines, might more fully protect the DNA from serum nucleases. Protamines are small, naturally occurring arginine-rich peptides whose interaction with DNA results in phosphate neutralization and DNA collapse.[43,44] To test the ability of protamine to protect the DNA, we incubated plasmid DNA and increasing concentrations of protamine in the presence of 20% active serum, with or without PBK10 at a standardized protein:DNA ratio (w/w) of 2. Complexes that are sensitive to nuclease activity were detected by the conversion of the plasmid from the supercoiled form to the open circle form, as described elsewhere.[45] Indeed, in the presence of serum alone, nearly all of the plasmid DNA, whether in the presence or absence of PBK10, is converted to nicked forms by 30 min at 37°C (Figure 5a). However, adding increasing concentrations of protamine preserves supercoiled DNA in the presence of active serum for up to 45 min, thus protecting the plasmid from nuclease-induced degradation.

**Table 1** Summary of PBK10-mediated gene delivery and expression

| *PBK10 (μM)* | *Serum (1%)* | *Theoretical charge ratio[a] (+:−)* | *Observed toxicity[b]* | *Relative transduction (fold over control[c])* |
|---|---|---|---|---|
| 3 | − | 1 | − | 412 |
| 5 | − | 1 | − | 371 |
| 15 | − | 1 | ++ | 634 |
| 7.5 | + | 0.5 | − | 7 |
| 15 | + | 1 | − | 29 |
| 75 | + | 5 | −/+ | 112 |
| 0.34 (+ protamine) | − | 13 | | 67 ± 13 |
| 0.34 (+ protamine) | + | 13 | | 53 ± 2 |

[a]Polylysine moiety residues and/or protamine cations: DNA phosphates.
[b]Percent of cells showing cytopathic effect: −, <1%, −/+, 1–10%, +, 10–50%; ++, >50%.
[c]GFP(+)/μg DNA/total cells) of treated over untreated cells.

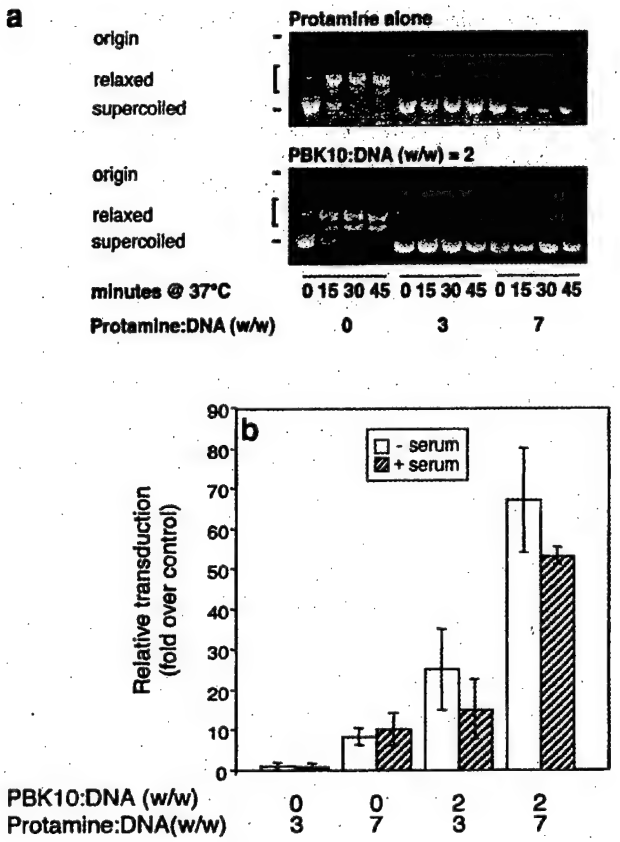


*Figure 5 Effect of serum on protamine-bound complexes. (a) DNA protection analysis. Plasmid is mixed with protamine and/or PBK10 at the indicated ratios, incubated in 20% active fetal bovine serum, and electrophoresed as described in Materials and methods. (b) Gene delivery and GFP expression in the presence of serum. DNA, protamine and/or PBK10 are mixed at the same ratios used in (a) and exposed to HeLa cells as described. Cells are exposed to mixes in serum-free media (white bars), or 1% fetal bovine serum-supplemented media (striped bars). Experiments were performed in triplicate, and relative transduction was determined by the percent of GFP-positive cells of treated cell populations over untreated cells. Error bars represent standard deviation.*

To test whether such protection of DNA from serum is effective in PBK10-mediated gene delivery, complexes were formed with increasing concentrations of protamine, and then exposed to HeLa cell monolayers in the presence or absence of 1% serum. It is evident by microscopy that the addition of protamine alone to DNA fails to produce GFP-positive cells (Figure 3e and f), suggesting that DNA condensation by protamine alone is not enough to produce high levels of gene delivery. However, the addition of protamine to PBK10-bound complexes enhances GFP expression by many-fold (Figure 3g and h), indicating that PBK10 is still required for efficient delivery. More importantly, there is little difference in GFP expression in the presence or absence of serum (Figure 5b and Table 1), indicating that protamine protects against serum-mediated inactivation of gene delivery. This suggests that the predominant factor inhibiting PBK10-mediated gene delivery seen in Figure 4b is the access of DNA to degradative nucleases found in serum.

### *PBK10-mediated gene delivery is specific*

Adding 1, 5 and 10 μg of protamine to constant amounts of plasmid produces negligible levels of GFP expression

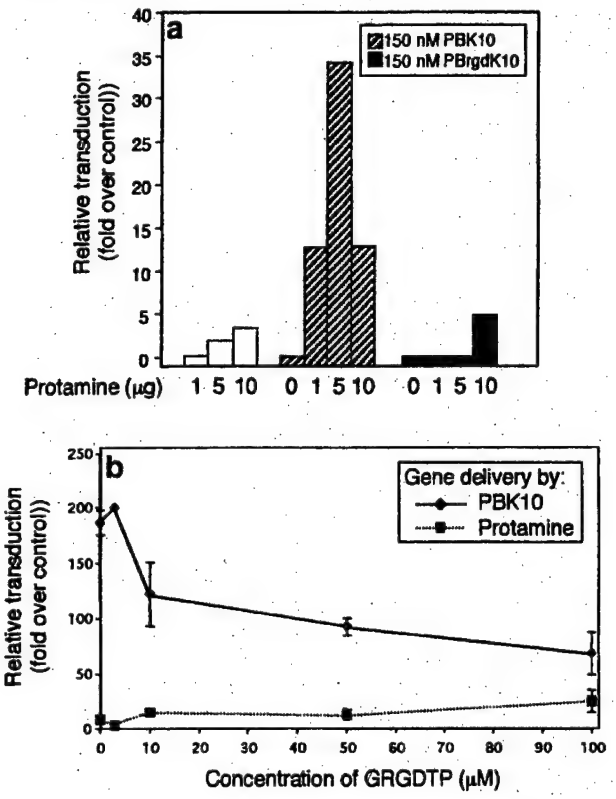


*Figure 6 Specificity of PBK10-mediated gene delivery. DNA (3.5 μg), protamine, and/or penton proteins are mixed and exposed to HeLa cells as described. (a) GFP expression is measured from cells treated with protamine condensed DNA (white bars), PBK10/protamine/DNA (striped bars), or PBrgdK10/protamine/DNA (gray bars). (b) Gene delivery and GFP expression in the presence of competing amounts of RGD peptide. GFP expression is measured from cells treated with protamine-condensed DNA (dotted line), or PBK10/protamine/DNA (solid line). Experiments were performed in triplicate, and relative transduction was determined by the percent of GFP-positive cells of treated cell populations over untreated cells. Error bars represent standard deviation.*

(Figure 6a, white bars), whereas adding PBK10 to all three protamine concentrations greatly enhances green fluorescence (Figure 6a, striped bars). The optimal ratios of PBK10 to protamine, however, determine the degree to which fluorescence is enhanced. Numerous studies show that a neutral to net positive charge promotes high cellular transfection of DNA complexes, presumably by preventing repulsion of DNA from negatively charged cell surface proteins.[46] It is demonstrated here, however, that PBK10 contributes more than mere charge neutralization to enhance gene delivery. At 1 μg concentrations of both PBK10 and protamine, the theoretical charge ratio (cations to DNA phosphates) is less than 1, yet PBK10 + protamine enhances GFP expression 52-fold higher than protamine alone (Figure 6a). GFP expression is enhanced an additional 2.6-fold by the inclusion of 5 μg of protamine with PBK10, which adds enough cations to theoretically neutralize all of the plasmid (Figure 6a). Excess protamine, however, is expected to reduce delivery if protamine competes with PBK10 for DNA binding. In agreement with this expectation, GFP expression is reduced 2.7-fold when PBK10 is incubated with 10 μg of protamine compared to 5 μg, despite the fact that there is a theoretical net positive charge. Nevertheless, at 10 μg

of protamine, GFP expression is still four-fold higher in the presence of PBK10 over protamine alone (Figure 6a). These results demonstrate that whereas charge neutralization is an important contributing factor for gene delivery, PBK10 is required for promoting high levels of delivery, presumably by directing the complexes to cellular receptors.

To confirm indeed that integrin receptor binding and endocytosis mediates cellular entry of the complexes described here, the RGD sequence of penton was mutated to produce a defective integrin binding site. The amino acid replacement, R340E, has been shown by others to severely reduce integrin-binding activity[36] and was, therefore, introduced here to produce the mutant penton, PBrgdK10. This mutant protein was tested for DNA binding activity by DNA mobility shift analysis (Figure 2b). PBrgdK10 produces similar plasmid retardation patterns as PBK10 by this assay, indicating that the mutation does not appear to impede DNA binding activity. Gene delivery by PBrgdK10 was tested on HeLa cells under the same conditions as described earlier. GFP expression is reduced to undetectable levels by PBrgdK10 compared to equivalent concentrations of PBK10 (Figure 6a, gray bars). Inclusion of 1 and 5 μg concentrations of protamine to PBrgdK10 complexes does not improve GFP expression. At 10 μg of protamine, GFP expression is enhanced only slightly, but remains 2.7-fold lower compared with the same concentration of protamine + PBK10.

Others have demonstrated that specific RGD-containing peptides interfere with penton binding to $\alpha_v$ integrins.[37] Similar peptides are used here, therefore, to provide further confirmation of specificity. A 300-fold molar excess of RGD-peptide to PBK10 reduces GFP expression by approximately 70% (Figure 6b, solid line). As protamine-mediated delivery comprises ≤10% of the GFP expression (Figure 6b, dotted line), LDV-mediated binding and internalization may account for the residual delivery. Taken together, these findings provide evidence that PBK10 directs the complexes to HeLa cells predominantly through the integrin-binding motif, RGD.

## Discussion

The utility of adenovirus capsid proteins for enhancing cell permeability has been implicated in previous studies. Co-administration of adenovirus with transferrin-mediated internalization of plasmid DNA resulted in enhanced plasmid gene expression, suggesting that adenoviral proteins promoted endosomal escape.[19] Direct conjugation of heat inactivated adenovirus to transferrin conjugates confirmed that adenovirus capsid proteins mediated the evasion of lysosomal degradation.[20,21] More recently, it has been shown that the penton protein alone is capable of enhancing vesicle lysis and could mediate the escape of plasmid DNA from endosomes.[36,47] Our data confirm that the penton possesses the capacity for translocation across cell membranes by direct linkage of a marker plasmid to the polylysine tract of PBK10 and subsequent expression of the marker gene in treated cells.

It has been shown that the 'dodecahedra' formed by Ad3 pentons are sufficient for endosome penetration and, when assembled with synthetic cationic peptides, can mediate cellular internalization of DNA.[47] Although the cavity formed by a 'dodecahedron' is too small to be occupied by plasmid DNA, the coating of these particles with cationic residues allows DNA binding over the surface of the molecule. In contrast, the placement of cationic residues at one end of the penton peptide sequence as described here presumably allows DNA binding at a localized region of the protein, with the intention of coating the DNA with penton protein molecules, rather than the other way around.

Protamine was found to enhance PBK10-mediated gene delivery in the presence of serum, presumably by protecting PBK10-bound DNA from serum nucleases. However, excess protamine reduces gene delivery. This is likely due to the competition of higher protamine with PBK10 for binding DNA. Therefore, the optimized ratios of PBK10/protamine/DNA that were chosen provide both protection from serum and efficient gene delivery. We name these PBK10/protamine/DNA complexes '3PO'.

Gene delivery by 3PO can be compared with that mediated by a recombinant adenovirus expressing GFP (Ad5-GFP). At 100 pmoles of PBK10 (approximately $10^9$ 3PO complexes), transduction efficiency of HeLa cells, as measured by GFP expression, is comparable to that of approximately $5 \times 10^6$ viral particles (data not shown). On the other hand, approximately $10^9$ viral particles are required to obtain similar levels of infection of HUVEC cells, which lack CAR, but express $\alpha_v\beta_3$ integrins.[48] This suggests that viral entry mediated by penton-integrin binding is 1000-fold less efficient than that mediated by fiber–CAR interaction. Thus, it seems that the 3PO complexes are as efficient in DNA delivery as adenoviruses in the absence of CAR receptors. It will be interesting to see, therefore, whether the transduction efficiency by 3PO will be enhanced by the addition of fiber protein. The optimized 3PO complexes described here contain, theoretically, 90 molecules of PBK10 per plasmid DNA. Interestingly, this number closely coincides with the number of pentons assembled per Ad5 capsid (60 pentons/capsid).

There are several advantages to using the penton capsid protein as opposed to the whole virus for gene delivery. One is that the size of the delivered gene is not constrained by a viral capsid, making this system flexible enough to accommodate potentially any size gene. Previous studies have shown that the use of a polylysine moiety binds to a wide range of nucleic acid sizes, from 564 base pairs to 48 kilobases, thus it is conceivable that the vector system presented here may accommodate a comparable range of gene sizes.[1,20] Additionally, 3PO complexes might have an advantage in delivery to tissues that lack CAR, as they will not be sequestered by CAR-expressing cells. Although the adenovirus capsid elicits a neutralizing immune response,[49,50] its efficient cell entry mechanism remains a desirable feature for gene delivery to both dividing and nondividing cells. A vector containing only the penton protein as opposed to the whole capsid is expected to elicit a much less potent immune response in comparison to the whole virus. It has been well established that *de novo* synthesis of viral proteins after infection produces a cytolytic T cell response to infected cells.[50–56] However, lack of all viral genes and other structural proteins should greatly minimize both the immunogenicity and toxicity of these vectors compared with current adenoviral vectors. In addition, protein-based delivery avoids many concerns associated

with using virus vectors, such as potential viral recombination. Finally, these 3PO complexes make use of the best features of the adenovirus capsid for efficient cell binding and internalization, while avoiding the need to produce high titers of actual virus.

## Materials and methods

### Cells, plasmids and peptides

293 and HeLa cells were maintained in DMEM, 10% fetal bovine serum, at 37°C, 5% $CO_2$. The reporter plasmid, pGFPemd-cmv [R] control vector (Packard Instrument Company, Meriden, CT, USA), was used for gene delivery assays. The RGD-containing peptide (GRGDTP) was obtained from Sigma.

### DNA constructs

A common 5′ oligonucleotide primer containing the sequence 5′-ATCGAAGGATCCATGCGG CGCGCGGCG ATGTAT-3′ was used to amplify both wild-type and lysine-tagged penton sequences from a pJM17 adenoviral genome template. The sequences of the 3′ primers are 5′-GCATCAGAATTCTCAAAAAGTGCGGCTCGATAG-3′ (wild-type penton) and 5′-CATGAATTCATTTTTTT TTTTTTTTTTTTTTTTTTTTTTTTAAAAGTGCGGCTCGA-TAGGA-3′ (lysine-tagged penton). A *Bam*HI restriction site was introduced in the 5′ primer and an *Eco*RI restriction site was introduced in the 3′ primers for in-frame insertion of both the wild-type and lysine-tagged pentons into the pRSET-A bacterial expression plasmid (Invitrogen, Carlsbad, CA, USA). This plasmid expresses the recombinant protein as an N-terminally histidine-tagged fusion for affinity purification by nickel chelate affinity chromatography.

### Protein expression and purification from bacteria

Overnight cultures of BL21(DE3)pLysS (Novagen, Madison, WI, USA) bacterial transformants were innoculated 1:50 in LB containing 0.1 mg/ml ampicillin and 0.034 mg/ml chloramphenicol. At OD 600 ~0.6, cultures were induced with 1 mM IPTG and grown 4 more h at 37°C with shaking. Cultures were harvested and pelleted. Cell pellets were resuspended in lysis buffer (50 mM Na-phosphate, pH 8.0; 500 mM NaCl; 5–10 mM imidazole; 1 mM phenylmethylsulfonyl fluoride) and lysed by addition of 0.1% Triton X-100 and one cycle of freeze–thawing. Supernatants were recovered, added to Ni-NTA resin (Qiagen, Valencia, CA, USA) pre-equilibrated in lysis buffer, and incubated for 1 h on ice. The resin containing bound protein was washed with 10 ml of lysis buffer, then 6 ml of a solution of 50 mM Na-phosphate, pH 8.0; 500 mM NaCl; 60 mM imidazole, and protein was eluted with 2 ml of a solution of 50 mM Na-phosphate, pH 8.0; 500 mM NaCl; 400 mM imidazole. Proteins were desalted on YM-10 nominal molecular weight limit spin columns (Millipore, Bedford, MA, USA) and their concentrations measured using the BioRad protein quantitation assay.

### Protein detection

Denaturing and nondenaturing polyacrylamide gel electrophoresis was performed in a discontinuous gel buffer system as previously described.[57] Nondenaturing gel electrophoresis differed by eliminating SDS from gels, running buffer, and sample buffer, and by not boiling the samples before electrophoresis. Proteins were electrically transferred on to nitrocellulose using 39 mM glycine, 48 mM Tris-HCl, 0.0375% SDS, 20% methanol in a BioRad semi-dry transfer cell set at constant voltage (15 V) for 30 min. Blots were blocked with 3% milk in PBS. Anti-His Tag antisera (Sigma, St Louis, MO, USA) was used at a 1:3000 dilution in blocking buffer. Anti-Ad5 antisera (Access Bio Medical, San Diego, CA, USA) was used at a 1:8000 dilution in blocking buffer. Antibody–antigen complexes were detected by incubation with horseradish peroxidase (HRP) conjugated secondary antibodies (Sigma), reaction with chemiluminescence detection reagents, and exposure to film (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

### Cell binding assay

Cells were treated with GFP-tagged proteins as described elsewhere.[42] Briefly, 293 cells grown to 75% confluency were lifted with phosphate-buffered saline (PBS) supplemented with 2 mM EDTA, centrifuged, washed, then added to PBS containing GFP-tagged proteins and 3% milk to reduce non-specific binding. After a 1 h incubation on ice, cells were centrifuged, washed thoroughly, resuspended in 0.5 ml PBS and measured by FACS scan.

### DNA mobility shift assays

A nonspecific oligonucleotide sequence (30 nucleotides) served as a nucleic acid probe for testing the electrostatic coupling of the lysine-tagged penton to nucleic acids. The oligonucleotide was radiolabelled with gamma (32P)dATP using the forward reaction enzyme activity of T4 kinase (Life Technologies, Rockville, MD, USA) following standard protocol, and unincorporated label was removed using a Nucleotide Purification Kit (Qiagen). Various concentrations of protein and oligonucleotide probe were incubated together in 15 μl total volumes in the presence of HEPES-buffered saline (HBS; 150 mM NaCl, 20 mM HEPES, pH 7.3) for 30 min at room temperature. Five microliters of sample dye (30% glycerol in water and bromophenol blue) were added, then each reaction was loaded on to a 5% polyacrylamide vertical gel pre-run in 1 × tris-glycine buffer for 20 min. The gel was run at room temperature at about 180 V for approximately 2 h. For the plasmid mobility shift assay, plasmid DNA was mixed with proteins at the indicated ratios in HBS for 30 min at room temperature in a total volume of 10 μl. After incubation, 2 μl of sample dye was added to the mixtures, mixes were loaded on a 0.8% agarose gel, and electrophoresed at 50 V. Gels were stained with ethidium bromide after electrophoresis to visualize DNA bands.

### DNA protection assay

Plasmid and proteins were added together at various ratios in a total volume of 20 μl and incubated at room temperature for 30 min. Four microliters of active (non-heat inactivated) fetal bovine serum were added to each mix and mixtures were incubated at 37°C. SDS at a 1% final concentration was added to each mix to release the proteins from the DNA. Four microliters of sample dye was added to each mix, and mixtures were electrophoresed on agarose gels as described earlier.

### Cell delivery assays

Cells were grown on 24-well dishes to approximately 75% confluency. Proteins and plasmids (3.5 or 5 μg) were

incubated together at various ratios at room temperature for 30 min in 0.3 ml of adhesion buffer (DMEM, 2 mM $MgCl_2$, 20 mM HEPES), then added to cell monolayers. Cells were exposed to mixes for 3–5 h at 37°C, 5% $CO_2$, before the addition of complete serum. For FACS analysis, cells were trypsinized 3 days after treatment, centrifuged at 500 *g*, and resuspended in 0.5 ml of phosphate buffered saline (PBS). In other assays, cells were trypsinized and counted using a hemocytometer under UV light microscopy to determine the percentage and absolute numbers of GFP-positive cells.

### *UV microscopy*

An Olympus (Lake Success, NY, USA) IMT-2 inverted microscope fitted with an FITC filter was used to visualize GFP-positive cells. Photomicrographs were taken at 4 × and 10 × magnification.

### *FACS analysis*

Where indicated, 5000–10000 events are counted on a Becton Dickinson FAC Scan analyzer (Becton Dickinson, Franklin Lakes, NJ, USA) using a 15 mW air-cooled argon laser set at 488 nm and recorded with a 530 nm emission filter in the FL1 emission channel. Cell populations are represented on a FACS histogram plotting green fluorescence intensity on a logarithmic scale against cell number. Fluorescence intensity of cell populations is indicated by a shift to the right of the histographical plots of treated cells. Fluorescence enhancement was determined by obtaining the number of gated fluorescent events for untreated and treated cells.

## Acknowledgements

We thank Toshiaki Shichinohe for the Ad5-GFP assay. We are grateful to the following people for ongoing discussions and support: Xinhua Chen, Gene Chung, John Daoud, Yasuo Hamamori, John Hwang, Tatsuya Iso, Kimi Kong, Vivian Leung, Jochen Muller-Ehmsen, Alex Oxyzoglou, Coralie Poizat, Tsuyoshi Sakoda, Vittorio Sartorelli, Terry Saluna, David Tinsley, and Hung-Yi Wu. Grants to LHK from the Susan G Komen Breast Cancer foundation, the Department of Defense, and the National Institutes of Health (CA59318–07) and a fellowship to LKM-K from the National Institutes of Health (HL09638–02) supported this work.

## References

1 Wagner E *et al*. Transferrin-polycation conjugates as carriers for DNA uptake into cells. *Proc Natl Acad Sci USA* 1990; **87**: 3410–3414.
2 Wagner E *et al*. DNA-binding transferrin conjugates as functional gene-delivery agents: synthesis by linkage of polylysine or ethidium homodimer to the transferrin carbohydrate moiety. *Bioconj Chem* 1991; **2**: 226–231.
3 Wagner E *et al*. Transferrin-polycation-DNA complexes: the effect of polycations on the structure of the complex and DNA delivery to cells. *Proc Natl Acad Sci USA* 1991; **88**: 4255–4259.
4 Plank C *et al*. Gene transfer into hepatocytes using asialoglycoprotein receptor mediated endocytosis of DNA complexed with an artificial tetra-antennary galactose ligand. *Bioconj Chem* 1992; **3**: 533–539.
5 Chiou HC *et al*. Enhanced resistance to nuclease degradation of nucleic acids complexed to asialoglycoprotein-polylysine carriers. *Nucleic Acids Res* 1994; **22**: 5439–5446.
6 McKee TD *et al*. Preparation of asialoorosomucoid-polylysine conjugates. *Bioconj Chem* 1994; **5**: 306–311.
7 Chowdhury NR *et al*. Fate of DNA targeted to the liver by asialoglycoprotein receptor-mediated endocytosis *in vivo*. Prolonged persistence in cytoplasmic vesicles after particle hepatectomy. *J Biol Chem* 1993; **268**: 11265–11271.
8 Stankovics J *et al*. Overexpression of human methylmalonyl CoA mutase in mice after *in vivo* gene transfer with asialoglycoprotein/polylysine/DNA complexes. *Hum Gene Ther* 1994; **5**: 1095–1104.
9 Zenke M *et al*. Receptor-mediated endocytosis of transferrin-polycation conjugates: an efficient way to introduce DNA into hematopoietic cells. *Proc Natl Acad Sci USA* 1990; **87**: 3655–3659.
10 Harris CE *et al*. Receptor-mediated gene transfer to airway epithelial cells in primary culture. *Am J Resp Cell Molec Biol* 1993; **9**: 441–447.
11 Schmidt M *et al*. Suppression of metastasis formation by a recombinant single chain antibody-toxin targeted to full-length and oncogenic variant EGF receptors. *Oncogene* 1999; **18**: 1711–1721.
12 Fominaya J, Wels W. Target cell-specific DNA transfer mediated by a chimeric multidomain protein. Novel non-viral gene delivery system. *J Biol Chem* 1996; **271**: 10560–10568.
13 Fomianaya J, Uherek C, Wels W. A chimeric fusion protein containing transforming growth factor-α mediates gene transfer via binding to the EGF receptor. *Gene Therapy* 1998; **5**: 521–530.
14 Wagner E. Ligand–polycation conjugates for receptor-targeted gene transfer. In: Huang M-CHL, Wagner E (eds). *Nonviral Vectors for Gene Therapy*. Academic Press: San Diego, 1999, pp 207–227.
15 Zhou X, Huang L. DNA transfection mediated by cationic liposomes containing lipopolylysine: characterization and mechanism of action. *Biochim Biophys Acta* 1994; **1189**: 195–203.
16 Zabner J *et al*. Cellular and molecular barriers to gene transfer by a cationic lipid. *J Biol Chem* 1995; **270**: 18997–19007.
17 Gottschalk S *et al*. A novel DNA–peptide complex for efficient gene transfer and expression in mammalian cells. *Gene Therapy* 1996; **3**: 48–57.
18 Fisher KJ, Wilson JM. The transmembrane domain of diphtheria toxin improves molecular conjugate gene transfer. *Biochem J* 1997; **321**: 49–58.
19 Curiel DT *et al*. Adenovirus enhancement of transferrin-polylysine-mediated gene delivery. *Proc Natl Acad Sci USA* 1991; **88**: 8850–8854.
20 Cotten M *et al*. High-efficiency receptor-mediated delivery of small and large (48 kilobase) gene constructs using the endosome-disruption activity of defective or chemically inactivated adenovirus particles. *Proc Natl Acad Sci USA* 1992; **89**: 6094–6098.
21 Wagner E *et al*. Coupling of adenovirus to transferrin-polylysine/DNA complexes greatly enhances receptor-mediated gene delivery and expression of transfected genes. *Proc Natl Acad Sci USA* 1992; **89**: 6099–6103.
22 Michael SI, Curiel DT. Strategies to achieve targeted gene delivery via the receptor-mediated endocytosis pathway. *Gene Therapy* 1994; **1**: 223–232.
23 Kozarsky KF, Wilson JM. Gene therapy: adenovirus vectors. *Curr Opin Genet Dev* 1993; **3**: 499–503.
24 Greber UF *et al*. Stepwise dismantling of adenovirus 2 during entry into cells. *Cell* 1993; **75**: 477–486.
25 Devaux C *et al*. Structure of adenovirus fibre. I. Analysis of crystals of fibre from adenovirus serotypes 2 and 5 by electron microscopy and X-ray crystallography. *J Molec Biol* 1990; **215**: 567–588.
26 Stewart PL *et al*. Image reconstruction reveals the complex molecular organization of adenovirus. *Cell* 1991; **67**: 145–154.
27 Boudin ML, Boulanger P. Assembly of adenovirus penton base and fiber. *Virology* 1982; **116**:589–604.
28 Bergelson JM *et al*. Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. *Science* 1997; **275**: 1320–1323.
29 Neumann R, Chroboczek J, Jacrot B. Determination of the nucleotide sequence for the penton-base gene of human adenovirus type 5. *Gene* 1988; **69**: 153–157.

30 Mathias P *et al.* Multiple adenovirus serotypes use $\alpha_v$ integrins for infection. *J Virol* 1994; **68**: 6811–6814.
31 Goldman MJ, Wilson JM. Expression of alpha v beta 5 integrin is necessary for efficient adneovirus-mediated gene transfer in the human airway. *J Virol* 1995; **69**: 5951–5958.
32 FitzGerald DJ *et al.* Adenovirus-induced release of epidermal growth factor and pseudomonas toxin into the cytosol of KB cells during receptor-mediated endocytosis. *Cell* 1983; **32**: 607–617.
33 Varga MJ, Weibull C, Everitt E. Infectious entry pathway of adenovirus type 2. *J Virol* 1991; **65**: 6061–6070.
34 Svensson U. Role of vesicles during adenovirus 2 internalization into HeLa cells. *J Virol* 1985; **55**: 442–449.
35 Seth P *et al.* Evidence that the penton base of adenovirus is involved in potentiation of toxicity of Pseudomonas exotoxin conjugated to epidermal growth factor. *Mol Cell Biol* 1984; **4**: 1528–1533.
36 Karayan L *et al.* Structural and functional determinants in adenovirus type 2 penton base recombinant protein. *J Virol* 1997; **71**: 8678–8689.
37 Wickham TJ *et al.* Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ promote adenovirus internalization but not virus attachment. *Cell* 1993; **73**: 309–319.
38 Komoriya A *et al.* The minimal essential sequence for a major cell type-specific adhesion site (CS1) within the alternatively spliced type III connecting segment domain of fibronectin is leucine-aspartic acid-valine. *J Biol Chem* 1991; **266**: 15075–15079.
39 Wickham TJ, Carrion ME, Kovesdi I. Targeting of adenovirus penton base to new receptors through replacement of its RGD motif with other receptor-specific peptide motifs. *Gene Therapy* 1995; **2**: 750–756.
40 Novelli A, Boulanger PA. Deletion analysis of functional domains in baculovirus-expressed adenovirus type 2 fiber. *Virology* 1991; **185**: 365–376.
41 Karayan L *et al.* Oligomerization of recombinant penton base of adenovirus type 2 and its assembly with fiber in baculovirus-infected cells. *Virology* 1994; **202**: 782–785.
42 Medina-Kauwe LK, Leung V, Wu L, Kedes L. Assessing the binding and endocytosis activity of cellular receptors using GFP-ligand fusions. *BioTechniques* 2000; **29**: 602–609.
43 Prieto MC, Maki AH, Balhorn R. Analysis of DNA–protamine interactions by optical detection of magnetic resonance. *Biochemistry* 1997; **36**: 11944–11951.
44 Warrant RW, Kim SH. alpha-Helix–double helix interaction shown in the structure of protamine–transfer RNA complex and a nucleoprotamine model. *Nature* 1978; **271**: 130–135.
45 Gao X, Huang L. Potentiation of cationic liposome-mediated gene delivery by polycations. *Biochemistry* 1996; **35**: 1027–1036.
46 Rolland AP. From genes to gene medicines: recent advances in nonviral gene delivery. *Crit Rev Ther Drug Carr Syst* 1998; **15**: 143–198.
47 Fender P *et al.* Adenovirus dodecahedron, a new vector for human gene transfer. *Nat Biotechnol* 1997; **15**: 52–56.
48 Shichinohe T *et al.* Development of lentiviral and adenoviral vectors for anti-angiogenic gene delivery. *Cancer Gene Ther* 2001 (submitted).
49 Schneider MD, French BA. The advent of adenovirus. Gene therapy for cardiovascular disease. *Circulation* 1993; **88**: 1937–1942.
50 Yang JP, Huang L. Direct gene transfer to mouse melanoma by intratumor injection of free DNA. *Gene Therapy* 1996; **3**: 542–548.
51 Yang Y, Ertl HC, Wilson JM. MHC class I-restricted cytotoxic T lymphocytes to viral antigens destroy hepatocytes in mice infected with E1-deleted recombinant adenoviruses. *Immunity* 1994; **1**: 433–442.
52 Yang Y, Wilson JM. Clearance of adenovirus-infected hepatocytes by MHC class I-restricted $CD4^+$ CTLs *in vivo*. *J Immunol* 1995; **155**: 2564–2570.
53 Yang Y *et al.* Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses. *J Virol* 1995; **69**: 2004–2015.
54 Yang Y, Greenough K, Wilson JM. Transient immune blockade presents formation of neutralizing antibody to recombinant adenovirus and allows repeated gene transfer to mouse liver. *Gene Therapy* 1996; **3**: 412–420.
55 Dai Y *et al.* Cellular and humoral immune responses to adenoviral vectors containing factor IX gene: tolerization of factor IX and vector antigens allows for long-term expression. *Proc Natl Acad Sci USA* 1995; **92**: 1401–1405.
56 Zsengeller ZK *et al.* Persistance of replication-deficient adenovirus-mediated gene transfer in lungs of immune-deficient (nu/nu) mice. *Hum Gene Ther* 1995; **6**: 457–467.
57 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; **227**: 680–685.

# Research Report

# Assessing the Binding and Endocytosis Activity of Cellular Receptors Using GFP-Ligand Fusions

*BioTechniques 29:602-609 (September 2000)*

**Lali K. Medina-Kauwe, Vivian Leung, Louis Wu and Larry Kedes**
University of Southern California Keck School of Medicine, Los Angeles, CA, USA

**ABSTRACT**

*We have developed a simple scheme for characterizing ligand-receptor binding and post-binding activity on living cells. Our approach makes use of green fluorescent protein (GFP) as an auto-fluorescent tag to label protein ligands. We have constructed GFP-tagged ligands that can be expressed in bacteria as soluble fusion proteins. A cell-binding assay using fluorescence-activated cell sorting (FACS) demonstrates that GFP-tagged proteins retain their wild-type receptor-binding specificity. Using this assay, we measure ligand binding on unfixed cells and demonstrate receptor specificity using specific competitors. To determine the ability of receptor targets to internalize, we developed a second FACS-based assay to detect the rate and percentage of internalized ligand in living cells. Noninternalizing control ligands and fluorescence microscopy of treated cells confirm that our assay is reliable for determining receptor internalization activity.*

## INTRODUCTION

Current methods for studying receptor binding and internalization commonly use radioactively labeled ligands or time-consuming fixation protocols that may distort both cells and proteins. We developed two simple nonradioactive assays for characterizing ligand-receptor binding and post-binding activity on unfixed, viable cells using the jellyfish *Aequorea victoria* green fluorescent protein (GFP) (6,7,34).

Because of its inherent fluorescence, GFP is ideal for experimental assays and detection methods on live cells (10,11, 18,22). The isolation and cloning of the GFP gene has allowed the production of recombinant protein for biochemical analyses (8,9,18,24,29), and recombinant GFP appears to retain its fluorescence and stability when produced as a fusion protein with foreign sequences. We have constructed recombinant genes encoding GFP fused to ligand proteins to study receptor-ligand interactions. The use of GFP in this capacity allows direct detection of ligand-receptor interactions while avoiding the need for additional biochemical treatments to detect activity, thus shortening and simplifying certain experimental procedures.

One of the ligand proteins that we use in our assays, heregulin-α, binds with high affinity to heterodimers of HER2/3 or HER2/4 receptor subunits, which are overexpressed on certain mammary tumor cell lines (1,3,4,12, 17,25–27,35). The binding kinetics between heregulin isomers and their target receptors have been well characterized using classical receptor-binding techniques, and the internalization activities of the receptor subunits have been studied (5,16,19–21,28,30,31). Here, we demonstrate that GFP can be used to monitor receptor binding and cellular internalization of the heregulin ligand under real-time conditions, and these activities can be measured using fluorescence-activated cell sorting (FACS).

## MATERIALS AND METHODS

### Cell Lines

The human breast cancer cell line MDA-MB-453 has been adapted to Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). HeLa cells are also maintained in the same media. Cells are grown at 37°C in the presence of 5% $CO_2$.

### Construction of Plasmids

Oligonucleotide primers were used to amplify the region encoding the receptor binding domains of heregulin-$\alpha_1$ (eHRG) and adenovirus sero-type 5 fiber protein (Knob) by PCR. Restriction sites for cloning were introduced by both sets of primers. Both DNA fragments were cut and ligated into pRSET™ A plasmids (Invitrogen, Carlsbad, CA, USA) that we modified by incorporating a cDNA encoding GFP into the multiple cloning region. Our GFP fragment was made by PCR amplification of the coding sequence of GFP minus the stop codon from the pEGFP-N1 plasmid (Clontech Laboratories, Palo Alto, CA, USA). Our ligand inserts were cloned just downstream of the GFP region, placing GFP at the amino (N)-terminus of the fusion protein. Expression from our pRSET-GFP plasmids produces GFP-eHRG

and GFP-Knob proteins, respectively. We also constructed plasmids without GFP inserts to produce untagged eHRG and Knob proteins. All proteins were made as an N-terminal histidine-tagged fusion for purification by metal chelate affinity chromatography. The histidine tag was not removed from our proteins.

### Protein Production

Plasmid constructs were transformed into BL21(DE3)pLysS (Novagen, Madison, WI, USA) competent bacteria and grown in LB supplemented with 0.05 mg/mL ampicillin and 0.034 mg/mL chloramphenicol. Transformants were grown to an OD600 of 0.5-1 and induced with 1 mM isopropyl-β-D-thiogalactoside (IPTG). After 3 h incubation, cells were collected and lysed by freeze-thaw in the presence of lysis buffer (50 mM sodium phosphate, monobasic, pH 8.0, 300 mM NaCl, 60 mM imidazole, 0.1% Triton® X-100 and 1 mM PMSF). Lysates were clarified by high-speed centrifugation and supernatants were incubated on ice for 1 h in the presence of Ni-NTA resin (Qiagen, Valencia, CA, USA) pre-equilibrated with wash buffer (50 mM sodium phosphate, monobasic, pH 8.0, 500 mM NaCl, 60 mM imidazole). Slurries were loaded onto polyprep chromatography columns (Bio-Rad Laboratories, Hercules, CA, USA), which were allowed to empty by gravity flow. Columns were washed with 20× bead volumes of wash buffer; protein was then eluted with elution buffer (50 mM sodium phosphate, monobasic, pH 8.0, 500 mM NaCl, 500 mM imidazole). Salt and imidazole were removed from protein samples by centrifugation in 10 000 molecular weight cutoff ultrafiltration columns (Pall Gelman Laboratory, Ann Arbor, MI, USA).

### Cell-Binding Assays

MDA-MB-453 cells maintained in a 24-well dish (about $10^5$ cells/well) were detached by treatment with PBS (13.7 mM NaCl, 0.27 mM KCl, 0.43 mM sodium phosphate, dibasic, 0.14 mM potassium phosphate, monobasic) supplemented with 2 mM EDTA. Cells were pelleted and washed four times with 1× PBS supplemented with 0.01% $Ca^{2+}$ and $Mg^{2+}$, then incubated by rocking for 1 h on ice with protein mixtures in blocking buffer (PBS and 3% milk) to reduce nonspecific binding. After incubation, cells were washed four times with PBS to remove unbound proteins, resuspended in 0.5 mL PBS and assayed by FACS. Competition assays were performed by repeating the protocol outlined for the cell-binding assay, but by co-incubating a non-GFP-tagged competitor with the protein mixtures.

### Internalization Assay

Cells were maintained on dishes, detached, incubated with protein mixes and washed as outlined above in the cell-binding assay. After the final wash, cells were placed at 37°C to allow internalization activity. They were collected by centrifugation (1000× *g*) at the indicated time points after warming, resuspended in 0.1 mL of 2 mg/mL trypsin, 2 mM EDTA and incubated at 37°C for 1 min with agitation to remove cell surface-bound proteins. Trypsinized cells were centrifuged and washed four times with PBS, then resuspended in 0.5 mL PBS and analyzed by FACS.

### FACS

Samples were counted using a FACScan™ (Becton Dickinson, Franklin Lakes, NJ, USA) with a 15 mW air-cooled argon laser set at an excitation wavelength of 488 nm and recorded with a 530-nm emission filter.

### Microscopy

Cells were viewed using an IMT-2 inverted UV light microscope (Olympus America, Melville, NY, USA). GFP fluorescence was observed using a FITC filter. Photomicrographs were taken at 40× magnification using an Olympus SC35 35 mm camera.

## RESULTS AND DISCUSSION

### GFP-Ligand Fusion Proteins Produced at High Levels in Bacteria

We modified two types of ligand to validate our assays and characterize receptor-binding activity. One of these was a 200-amino acid peptide (eHRG) containing an EGF-like motif that, alone, confers binding specificity to HER2-containing receptors (14,16). The version of ligand presented here has successfully directed the cell-specific binding of retroviral vectors when genetically engineered into the viral envelope (13), suggesting that heregulin binding is not impaired when produced as a fusion to foreign sequences. We

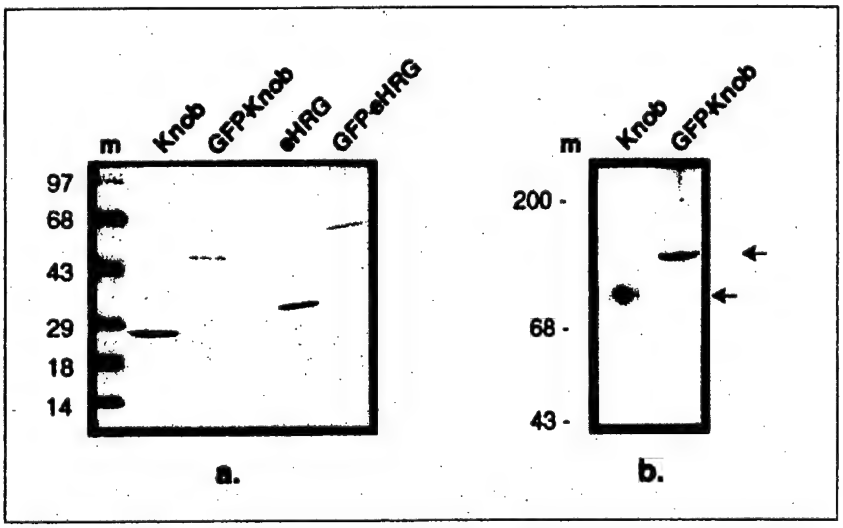


**Figure 1. Denaturing and non-denaturing PAGE analysis of recombinant proteins.** Sizes of marker proteins are given in kilodaltons to the left of each gel; m, protein size marker. Both gels are Coomassie stained. (a) SDS-PAGE analysis of affinity purified proteins. (b) Non-denaturing SDS-PAGE analysis of Knob and GFP-Knob. Arrows point to trimers.

also modified the C-terminal globular domain of the Ad5 Knob, which binds with high affinity to ubiquitous cellular receptors known as Coxsackievirus adenovirus receptors (CAR) (2). We expressed both peptides alone and as GFP-tagged versions (GFP-eHRG and GFP-Knob) in bacteria. All proteins were produced at high levels, were highly soluble and could be isolated to a high degree of purity by affinity chromatography. Figure 1a shows that these proteins migrate at their expected molecular weights under denaturing conditions.

The homotrimeric nature of the Knob protein has been well established (15, 33). To assess whether the GFP moiety affects Knob trimer assembly, we determined the molecular size of both GFP-tagged and untagged versions of the Knob (GFP-Knob and Knob, respectively) under native conditions. Using non-denaturing SDS-PAGE (23), we found that both Knob and GFP-Knob migrate at the expected size for trimers (Figure 1b), suggesting that the GFP segment does not interfere with oligomerization.

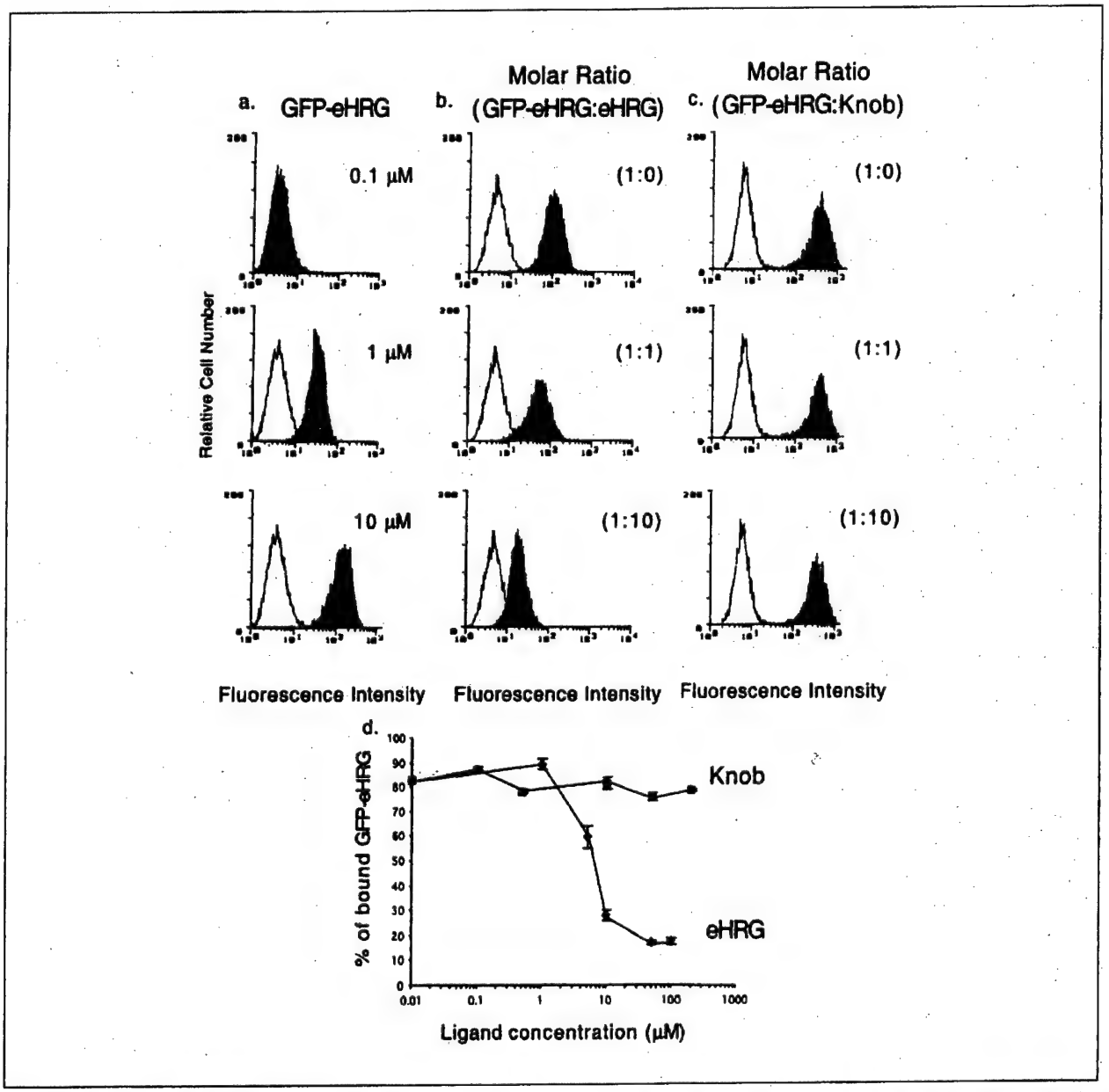


**Figure 2. FACS results of cell-binding assays on MDA-MB-453 cells.** Untreated cells are shown in white. Treated cells are shown in gray. Fluorescence intensity is shown on the x-axis using a logarithmic scale. (a) Dose-related fluorescence enhancement by the indicated concentrations of cell-bound GFP-eHRG. (b and c) Competition assays. Cells were incubated with GFP-eHRG in the presence of varying concentrations of non-GFP-tagged ligands: (b) eHRG or (c) Knob. (d) Summary of competition assays. The value of the mean population shift of cells treated with GFP-eHRG alone was set at 100%, indicating total bound protein. The values of the mean population shifts produced by the addition of eHRG (diamonds) or Knob (squares) were converted to a percentage of the total bound GFP-eHRG and plotted against the concentrations of each competitor.

## GFP-Ligand Fusions Retain Receptor-Binding Activity

The human breast cancer cell line MDA-MB-453, which overexpresses HER2-containing receptors, was used to test receptor binding of GFP-eHRG. Increasing concentrations of GFP-eHRG were incubated with MDA-MB-453 cells on ice in the presence of a blocking agent (3% milk powder) to reduce nonspecific binding. Cells were washed thoroughly to remove nonspecifically bound protein and analyzed by FACS. GFP-eHRG exhibits dose-dependent binding activity, as shown by a progressive increase in fluorescence shift of the treated populations of cells (Figure 2a).

We repeated the cell-binding assays in the presence of increasing amounts of untagged competitor (eHRG) to

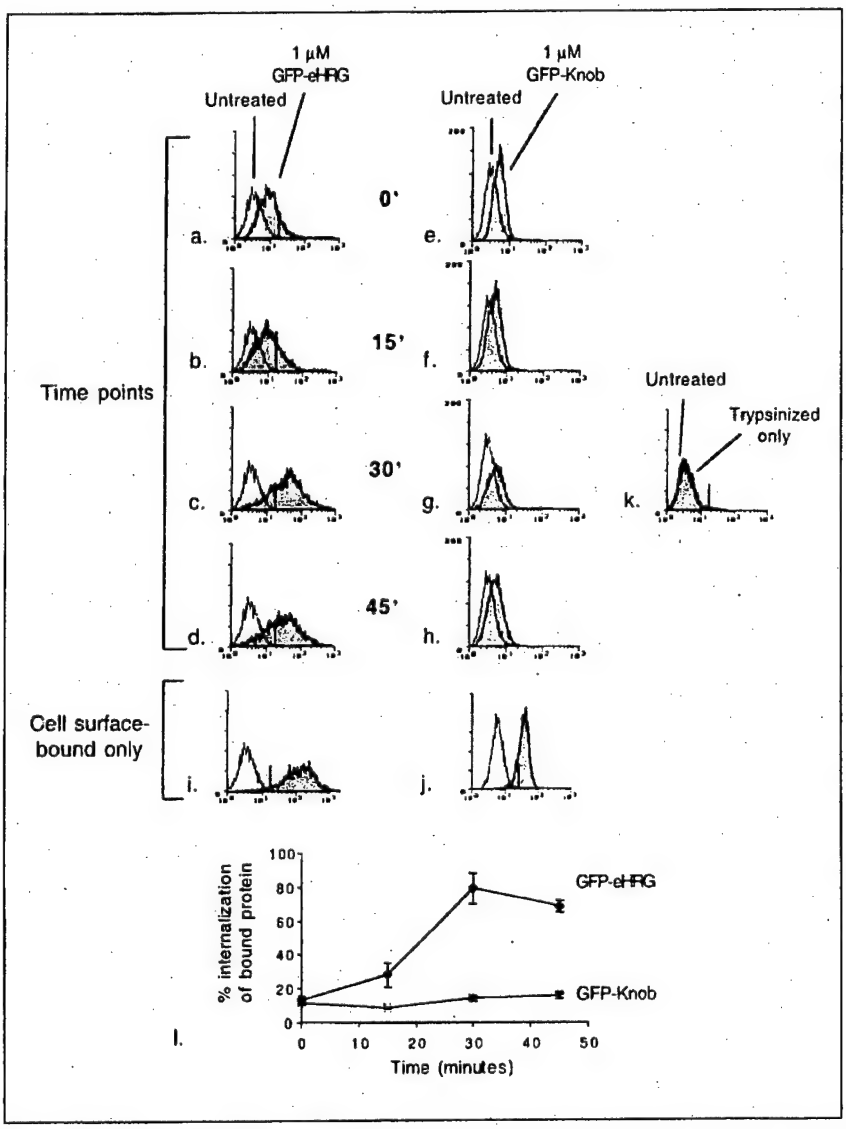


**Figure 3. FACS results of internalization assay.** Untreated MDA-MB-453 cells are shown in white. Treated MDA-MB-453 cells are shown in gray. (a–d and e–h) Fluorescence shift of the GFP-ligand treated cells collected at the indicated time points after warming. (i and j) Fluorescence shift of untrypsinized cells treated with the indicated GFP-ligands. (k) Untreated cells (white) and untreated trypsinized cells (gray). In k, the curves superimpose, showing that trypsin treatment alone does not produce an artifactual fluorescence shift. (l) Summary of internalization assay. The value of the mean population shift produced by the indicated GFP-ligand on untrypsinized cells (i and j) was set at 100%, indicating total bound protein. The values of the mean population shifts produced by the treated cells at each time point (a–d and e–h) were converted to a percentage of the total bound protein on untrypsinized cells.

determine whether the observed fluorescence shift is a result of specific ligand binding to cellular receptors and not caused by nonspecific binding to cell surfaces. The fluorescence shift resulting from GFP-eHRG binding is sequentially reduced in the presence of successively increasing concentrations of non-GFP-tagged eHRG (Figure 2b). To further confirm receptor-binding specificity, we tried to compete away GFP-eHRG binding with an unrelated protein (Knob). The fluorescence shift resulting from GFP-eHRG binding could not be reduced with excess concentrations of the unrelated competitor and so established the specificity of this cell-binding assay (Figure 2c).

Figure 2d summarizes the results of these competition assays and shows that the amount of bound GFP-ligand that is competed away inversely correlates with the concentration of untagged competitor. Similar results were obtained using GFP-Knob and Knob protein on HeLa cells. Thus, the GFP-tagged ligands retain specific binding to their respective receptor targets, indicating that the GFP-moiety does not interfere with the receptor-binding function.

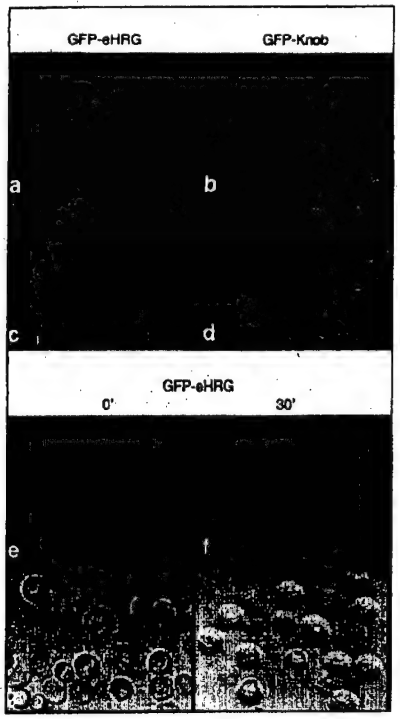


**Figure 4. Fluorescence microscopy of MDA MB-453 cells from internalization assay at 40× magnification under fluorescent light (a, b, e and f) and regular light (c, d, g and h).** (a–d) View of untrypsinized cells at 30 min after warming. (e–h) View of trypsinized cells at 0 min (e and g) and 30 min (f and h) after warming.

### Establishing a FACS-Based Assay for Internalization Activity

We modified the cell-binding assay to measure intracellular accumulation of the GFP-tagged proteins to determine whether the heregulin receptor internalizes after binding and, if so, its rate of internalization. First, we allowed MDA-MB-453 human breast cancer cells to bind GFP-eHRG on ice and washed the cells extensively to remove unbound proteins. The cells were then incubated at 37°C and collected at sequential time points after warming. Each cell harvest was immediately followed by treatment with trypsin/EDTA to remove residual cell surface-bound proteins and then analyzed by FACS. Internalized GFP is protected from trypsinization and so produces a fluorescence shift that is detectable by FACS. This assay approach predicts that a progressively increased accumulation of intracellular GFP should produce a progressively enhanced fluorescence shift in the treated cell population over time. In contrast, GFP-ligands continuously retained on the cell surface are removed by trypsin treatment and produce no fluorescence signal at any time point.

As shown in Figure 3, a–d, MDA-MB-453 cells treated with GFP-eHRG exhibit an increase in fluorescence shift over time, suggesting the accumulation of internalized GFP-eHRG. To verify our findings, we performed the same assay using GFP-Knob. It has previously been shown that the Knob receptor, CAR, binds the Knob with high affinity but does not internalize after ligand binding (32). We did not expect GFP-Knob to produce an increase in fluorescence shift over time because of its inability to internalize. Indeed, no such shift in fluorescence is produced when cells are treated with GFP-Knob (Figure 3, e–h), despite the high degree of binding on the surface of untrypsinized but thoroughly washed MDA-MB-453 cells (Figure 3j). Trypsinization of cells alone does not induce a fluorescence shift (Figure 3k). Figure 3l summarizes these findings and shows that of the total GFP-eHRG bound on the surface of MDA-MG-453 cells at 0 min, 80% is internalized by 30 min at 37°C, suggesting rapid internalization after binding.

Microscopic examination of our treated cells shows a clear difference in distribution of GFP-eHRG and GFP-Knob after performing the internalization assay. At 30 min after warming but before trypsinization, there are bright, punctate fluorescent foci resembling endosome-like compartments in the cells treated with GFP-eHRG (Figure 4a). This contrasts with the diffuse fluorescence observed on cells treated with GFP-Knob (Figure 4b). Under regular light, both sets of treated cells appear morphologically similar (Figure 4, c and d) and unchanged from the morphology of healthy, untreated MDA-MB-453 cells. A clear difference in fluorescence also emerges from trypsinized MDA-MB-453 cells at 0 and 30 min after warming. Although little fluorescence above background is apparent at 0 min (Figure 4e), many bright intracellular foci are seen at 30 min (Figure 4f). This pattern clearly shows that many GFP-eHRG proteins have become internalized by 30 min and so are protected from trypsin treatment.

It may be feasible to apply the methods presented here to a broader range of applications when testing ligand-receptor activity. For example, FACS may use GFP-tagged ligands in place of antibodies to identify and sort cells. Our assays may also be used to compare the binding and internalization of different ligand or receptor isomers or mutants. In addition, our approach may simplify the analysis of different structural mutations on receptor binding and internalization. The stability of GFP as a fusion protein allows a number of different applications for the study of ligand-receptor interactions under natural conditions and avoids the need to fix cells at a certain endpoint or treat biological samples with radioactive reagents.

## ACKNOWLEDGMENTS

We thank Nori Kasahara for providing the heregulin sequence and breast cancer cell lines. We are also grateful to the following people for ongoing

discussions and support: Xinhua Chen, Gene Chung, John Daoud, Yasuo Hamamori, John Hwang, Tatsuya Iso, Kimi Kong, Jochen Muller-Ehmsen, Alex Oxyzoglou, Coralie Poizat, Tsuyoshi Sakoda, Vittorio Sartorelli, David Tinsley and Hung-Yi Wu. This work was supported by grants to L.H.K. from the Susan G. Komen Breast Cancer foundation and the Department of Defense; a fellowship to L.K.M.-K. from the National Institutes of Health grant no. HL09638-02 supported this work.

## REFERENCES

1. **Bacus, S.S., C.R. Zelnick, G. Plowman and Y. Yarden.** 1994. Expression of the erbB-2 family of growth factor receptors and their ligands in breast cancers. Implication for tumor biology and clinical behavior. Am. J. Clin. Pathol. *102*:S13-S24.
2. **Bergelson, J.M., J.A. Cunningham, G. Droguett, E.A. Kurt-Jones, A. Krithivas, J.S. Hong, M. S. Horwitz, R.L. Crowell and R.W. Finberg.** 1997. Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. Science *275*:1320-1323.
3. **Carraway, K.L., III and L.C. Cantley.** 1994. A neu acquaintance for erbB3 and erbB4: a role for receptor heterodimerization in growth signaling. Cell *78*:5-8.
4. **Carraway, K.L., III, M.X. Sliwkowski, R. Akita, J.V. Platko, P.M. Guy, A. Nuijens, A.J. Diamonti, R.L. Vandlen et al.** 1994. The erbB3 gene product is a receptor for heregulin. J. Biol. Chem. *269*:14303-14306.
5. **Carraway, K.L, III and R.A. Cerione.** 1993. Fluorescent-labeled growth factor molecules serve as probes for receptor binding and endocytosis. Biochemistry *32*:12039-12045.
6. **Chalfie, M.** 1995. Green fluorescent protein. Photochem. Photobiol. *62*:651-656.
7. **Chalfie, M., Y. Tu, G. Euskirchen, W.W. Ward and D.C. Prasher.** 1994. Green fluorescent protein as a marker for gene expression. Science *263*:802-805.
8. **Crameri, A., E.A. Whitehorn, E. Tate, and W.P. Stemmer.** 1996. Improved green fluorescent protein by molecular evolution using DNA shuffling. Nat. Biotechnol. *14*:315-319.
9. **Delagrave, S., R. E. Hawtin, C.M. Silva, M.M. Yang and D.C. Youvan.** 1995. Red-shifted excitation mutants of the green fluorescent protein. BioTechnology *13*:151-154.
10. **Elliott, G. and P. O'Hare.** 1997. Intercellular trafficking and protein delivery by a herpes virus structural protein. Cell *88*:223-233.
11. **Epel, B.L., H.S. Padgett, M. Heinlein and R.N. Beachy.** 1996. Plant virus movement protein dynamics probed with a GFP-protein fusion. Gene *173*:75-79.
12. **Goldman, R., R.B. Levy, E. Peles and Y. Yarden.** 1990. Heterodimerization of the erbB-1 and erbB-2 receptors in human breast carcinoma cells: a mechanism for receptor transregulation. Biochemistry *29*:11024-11028.
13. **Han, X., N. Kasahara and Y.W. Kan.** 1995. Ligand-directed retroviral targeting of human breast cancer cells. Proc. Natl. Acad. Sci. USA *92*:9747-9751.
14. **Harris, A., M. Adler, J. Brink, R. Lin, M. Foehr, M. Ferrer, B.C. Langton-Webster, R.N. Harkins and S.A. Thompson.** 1998. Homologue scanning mutagenesis of heregulin reveals receptor specific binding epitopes. Biochem. Biophys. Res. Commun. *251*:220-224.
15. **Henry, L.J., D. Xia, M.E. Wilke, J. Deisenhofer and R.D. Gerard.** 1994. Characterization of the knob domain of the adenovirus type 5 fiber protein expressed in *Escherichia coli*. J. Virol. *68*:5239-5246.
16. **Holmes, W.E., M.X. Sliwkowski, R.W. Akita, W.J. Henzel, J. Lee, J.W. Park, D. Yansura, N. Abadi et al.** 1992. Identification of heregulin, a specific activator of p185erbB2. Science *256*:1205-1210.
17. **Hung, M.C., A. Matin, Y. Zhang, X. Xing, F. Sorgi, L. Huang and D. Yu.** 1995. HER-2/neu-targeting gene therapy—a review. Gene *159*:65-71.
18. **Katz, B.Z., D. Krylov, S. Aota, M. Olive, C. Vinson and K.M. Yamada.** 1998. Green fluorescent protein labeling of cytoskeletal structures-novel targeting approach based on leucine zippers. BioTechniques *25*:298-304.
19. **Lenferink, A.E., R. Pinkas-Kramarski, M.L. van de Poll, M.J. van Vugt, L.N. Klapper, E. Tzahar, H. Waterman, M. Sela et al.** 1998. Differential endocytic routing of homo- and hetero-dimeric ErbB tyrosine kinases confers signaling superiority to receptor heterodimers. EMBO J. *17*:3385-3397.
20. **Levkowitz, G., H. Waterman, E. Zamir, Z. Kam, S. Oved, W.Y. Langdon, L. Beguinot, B. Geiger and Y. Yarden.** 1998. c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. Genes Dev. *12*:3663-3374.
21. **Li, W., J.W. Park, A. Nuijens, M.X. Sliwkowski and G.A. Keller.** 1996. Heregulin is rapidly translocated to the nucleus and its transport is correlated with c-myc induction in breast cancer cells. Oncogene *12*:2473-2477.
22. **Moores, S.L., J.H. Sabry and J.A. Spudich.** 1996. Myosin dynamics in live Dictyostelium cells. Proc. Natl Acad. Sci. USA *93*:443-446.
23. **Novelli, A. and P.A. Boulanger.** 1991. Assembly of adenovirus type 2 fiber synthesized in cell-free translation system. J. Biol. Chem. *266*:9299-9303.
24. **Prasher, D.C., V.K. Eckenrode, W.W. Ward, F.G. Prendergast and M.J. Cormier.** 1992. Primary structure of the *Aequorea victoria* green-fluorescent protein. Gene *111*:229-233.
25. **Press, M.F., L.A. Jones, W. Godolphin, C.L. Edwards and D.J. Slamon.** 1990. HER-2/neu oncogene amplification and expression in breast and ovarian cancers. Prog. Clin. Biol. Res. *354A*:209-221.
26. **Slamon, D.J. and G.M. Clark.** 1988. Amplification of c-erbB-2 and aggressive human breast tumors? Science *240*:1795-1798.
27. **Slamon, D.J., G.M. Clark, S.G. Wong, W.J. Levin, A. Ullrich and W.L. McGuire.** 1987. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science *235*:177-182.
28. **Sliwkowski, M.X., G. Schaefer, R.W. Akita, J.A. Lofgren, V.D. Fitzpatrick, A. Nuijens, B.M. Fendly, R.A. Cerione et al.** 1994. Coexpression of erbB2 and erbB3 proteins reconstitutes a high affinity receptor for heregulin. J. Biol. Chem. *269*:14661-14665.
29. **Stearns, T.** 1995. Green fluorescent protein. The green revolution. Curr. Biol. *5*:262-264.
30. **Tzahar, E., G. Levkowitz, D. Karunagaran, L. Yi, E. Peles, S. Lavi, D. Chang, N. Liu et al.** 1994. ErbB-3 and ErbB-4 function as the respective low and high affinity receptors of all Neu differentiation factor/heregulin isoforms. J. Biol. Chem. *269*:25226-25233.
31. **Waterman, H., I. Sabanai, B. Geiger and Y. Yarden.** 1998. Alternative intracellular routing of ErbB receptors may determine signaling potency. J. Biol. Chem. *273*:13819-13827.
32. **Wickham, T.J., P. Mathias, D.A. Cheresh and G.R. Nemerow.** 1993. Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ promote adenovirus internalization but not virus attachment. Cell *73*:309-319.
33. **Xia, D., L.J. Henry, R.D. Gerard and J. Deisenhofer.** 1994. Crystal structure of the receptor-binding domain of adenovirus type 5 fiber protein at 1.7 A resolution. Structure *2*:1259-1270.
34. **Yang, F., L.G. Moss and G.N. Phillips, Jr.** 1996. The molecular structure of green fluorescent protein. Nat. Biotechnol. *14*:1246-1251.
35. **Yarden, Y. and R.A. Weinberg.** 1989. Experimental approaches to hypothetical hormones: detection of a candidate ligand of the neu protooncogene. Proc. Natl. Acad. Sci. USA *86*:3179-3183.

Received 2 November 1999; accepted 25 April 2000.

**Address correspondence to:**

Dr. Larry Kedes
*Institute for Genetic Medicine and Department of Biochemistry & Molecular Biology*
*University of Southern California Keck School of Medicine*
*2250 Alcazar Street CSC240*
*Los Angeles, CA 90033, USA*
*e-mail: kedes@hsc.usc.edu*

Gene Therapy (2001) 8, 1753–1761

www.nature.com/gt

**RESEARCH ARTICLE**

# Nonviral gene delivery to human breast cancer cells by targeted Ad5 penton proteins

LK Medina-Kauwe[1], M Maguire[1], N Kasahara[1,2] and L Kedes[1]

[1]*Institute for Genetic Medicine and Department of Biochemistry and Molecular Biology, University of Southern California Keck School of Medicine, Los Angeles, CA, USA; and* [2]*Department of Pathology, University of Southern California Keck School of Medicine, Los Angeles, CA, USA*

*The capsid proteins of adenovirus serotype 5 (Ad5) are key to the virus' highly efficient cell binding and entry mechanism. In particular, the penton base plays a significant role in both viral internalization and endosome penetration. We have produced an adenovirus penton fusion protein (HerPBK10) containing moieties for DNA transport and targeted delivery to breast cancer cells. HerPBK10 binds DNA through a polylysine appendage, while the EGF-like domain of the heregulin-$\alpha_1$ isoform is used as the targeting ligand. This ligand binds with high affinity to HER2/3 or HER2/4 heterodimers, which are overexpressed on certain aggressive breast cancers. In addition, this ligand is rapidly internalized after binding, thus adding to the utility of heregulin for targeting. HerPBK10 binds MDA-MB-453 breast cancer cells in a receptor-specific manner, and mediates the entry of a reporter plasmid in MDA-MB-453 cells in culture. Delivery can be competed by excess heregulin peptide, thus confirming receptor specificity. Importantly, the penton segment appears to contribute significantly to enhanced delivery. Complexes containing HerPBK10 and DNA have been optimized to provide targeted gene delivery to breast cancer cells in vitro. We demonstrate that delivery can be accomplished in the presence of serum, thus suggesting a potential use for in vivo delivery.* Gene Therapy (2001) **8**, 1753–1761.

**Keywords:** *penton; heregulin; adenovirus; gene delivery; breast cancer; nonviral*

## Introduction

Growing concerns about gene therapy vector safety have spurred the ongoing development of physically targeted gene delivery vehicles. Among the advantages to targeting gene therapy is the lack of undesirable delivery to tissues other than the target tissue. Additionally, physical targeting should require lower doses of vector in comparison to an untargeted vector, and presumably, reduce the immunogenicity and toxicity of the vector.

Despite the potent immune response elicited by adenovirus vectors,[1,2] the highly efficient cell entry mechanism of the virus remains a desirable feature for gene delivery to both dividing and nondividing cells. Particularly, the cell binding and entry functions imparted by the fiber and penton capsid proteins of adenovirus serotype 5 (Ad5) are a major reason why adenoviruses continue to be widely used as vehicles for gene transfer.[3,4] The antenna-like fiber proteins that protrude from each vertex of the icosahedral-shaped viral capsid bind with high affinity to ubiquitous cell surface Coxsackievirus adenovirus receptor (CAR) proteins.[5] This interaction initiates the infection mechanism of the virus, and is followed by the binding of the homopentameric penton base proteins, which lie at the base of each fiber, to cell surface integrins.[6] Integrin receptor-mediated endocytosis provides the cellular entry of the virus, but traps it in cellular endosomes. Endosome escape appears to be mediated by the penton, thus allowing entry of the virus to the cytosol.[7,8]

Presumed less toxic and immunogenic, nonviral vectors have been designed to mimic the receptor-mediated cell entry of adenoviruses. Early attempts to deliver genes by nonviral receptor-mediated endocytosis had fallen short of the goal of effective gene transfer, mainly because of the lack of cellular endosome escape. The inclusion of viral fusogenic peptides or endosomolytic agents with nonviral vectors has enhanced the gene delivery capacity of nonviral systems.[9–11] The adenovirus penton protein has been used for such a purpose. We have previously shown that a recombinant penton protein containing a decalysine sequence (PBK10) can bind DNA and mediate gene delivery into cells in culture in the absence of whole virus.[12] This delivery appeared to be mediated mainly through integrin receptor binding. When mixed at optimized ratios with plasmid DNA and the DNA condensing agent, protamine, effective gene delivery complexes were formed (PBK10/protamine/plasmid DNA) and have been designated 3PO. Here we describe the production of a targeted penton protein (HerPBK10) containing a receptor-specific ligand to direct the binding of similar complexes to certain breast cancer cells.

The ligand protein used here is the receptor-binding

*Correspondence: L Medina-Kauwe, Institute for Genetic Medicine and Department of Biochemistry and Molecular Biology, University of Southern California Keck School of Medicine, 2250 Alcazar Street, Los Angeles, CA 90033, USA*
Received 25 March 2001; accepted 31 August 2001

domain of heregulin-α. This ligand binds with high affinity to heterodimers of HER2/3 or HER2/4 receptor subunits, which are overexpressed on certain mammary tumor cell lines.[13–22] The binding kinetics between heregulin isomers and their target receptors have been well characterized using classical receptor binding techniques, and the internalization activities of the receptor subunits have been studied.[23–28] We have previously shown that the heregulin ligand is rapidly internalized after binding to MDA-MB-453 human breast cancer cells.[29] Here we show that by appending the heregulin ligand to the recombinant penton protein, we can direct the delivery of conjugated plasmid DNA specifically to these breast cancer cells by way of heregulin receptor binding and internalization, while preserving the endosomolytic function of the penton which enhances transduction efficiency.

## Results

### *Description and bacterial production of recombinant proteins*

We constructed recombinant genes encoding several specific proteins (Figure 1a). One of these constructs encodes the wild-type Ad5 penton (PB). A second construct encodes the Ad5 penton protein containing a carboxy (C)-terminal fusion of 10 lysines for binding DNA (PBK10). A third construct adds an amino (N)-terminal cell specific ligand to PBK10 to produce the targeted fusion protein, HerPBK10. We have also made the ligand alone (Her; also known as eHRG)[29] and as a C-terminal decalysine fusion protein (HerK10). Additionally, the ligand was produced as an N-terminal fusion to green fluorescent protein (GFP-Her; also known as GFP-eHRG).[29]

We have previously shown that PB, PBK10, and Her can be produced in bacteria as soluble proteins.[12,29] Using a similar bacterial expression system, we produced HerPBK10 and HerK10. The ligand peptide used here is derived from the receptor binding domain of heregulin and contains an EGF-like motif that confers binding specificity to HER2-containing receptors.[23,30] The version of ligand presented here has successfully directed the cell-specific binding of retroviral vectors when genetically engineered into the viral envelope.[31] We have also previously shown that heregulin receptor binding by this peptide is not impaired when fused to foreign sequences, such as GFP.[29]

Polyclonal antiserum specific to Ad5 capsid proteins recognizes all three penton proteins (HerPBK10, PBK10 and PB), thus confirming their identities (Figure 1b). Additional control proteins, Her, HerK10 and GFP-Her (not shown) were also highly expressed in bacteria and could be recognized by antibodies directed against the recombinant proteins. All proteins migrate at their expected molecular weights under denaturing conditions (Figure 1b). The polylysine tag of HerK10 adds 10 additional amino acids to the Her segment, therefore the molecular weight of HerK10 is only about 1 kDa higher than Her.

### *HerPBK10 binds DNA*

The polylysine tracts encoded by the recombinant penton constructs, PBK10 and HerPBK10, should impart DNA binding function to the fusion proteins by interacting

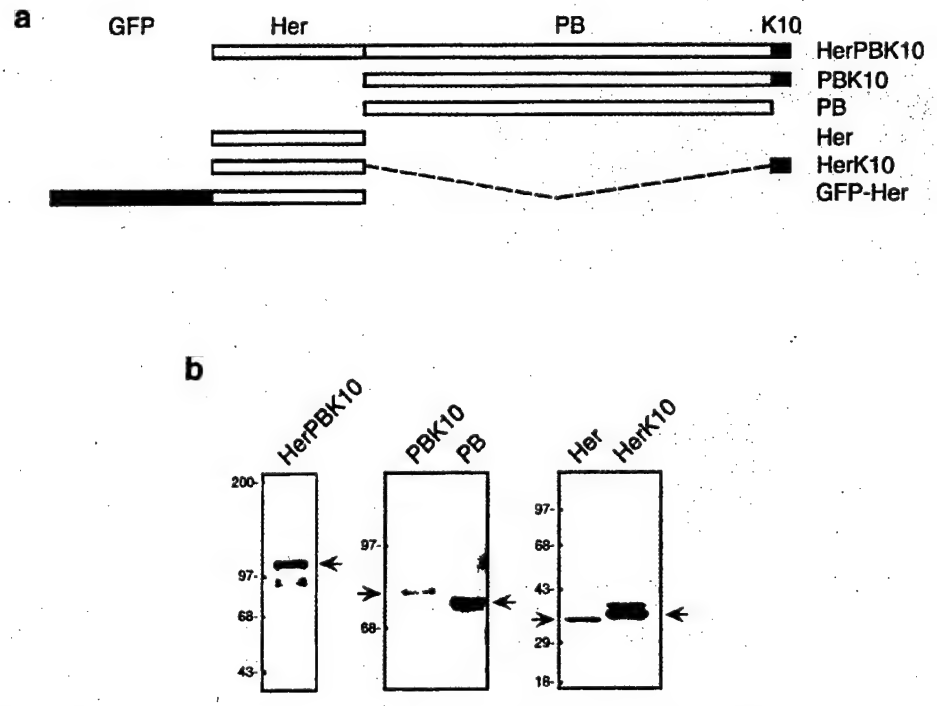


*Figure 1 Construction and production of recombinant proteins. (a) Schematic representation of proteins. Each bar represents the N to C terminal orientation of each protein (not drawn to scale). (b) Immunodetection of recombinant proteins. All proteins were electrophoresed under denaturing conditions. HerPBK10, PBK10 and PB were detected by a polyclonal antiserum directed against Ad5 capsid proteins (penton, hexon and fiber). Her and HerK10 were detected by an anti-histidine tag monoclonal antiserum.*

with the negatively charged phosphate backbone of nucleic acids. To determine DNA binding ability, increasing concentrations of protein were incubated with constant amounts of a 5 kb plasmid DNA (pGFPemd-cmv), and the resulting effect on DNA mobility analyzed on an agarose gel. At concentrations where the amount of polylysine completely binds, and thus neutralizes all of the DNA, the plasmid appears immobilized on the gel. We have previously shown that PBK10 immobilizes the plasmid at the predicted protein-to-DNA (w/w) ratio of 22.[12] The predicted ratio of HerPBK10 to plasmid to produce the same effect is 29, and our results agree with this prediction (Figure 2b). HerK10 exhibits a similar DNA mobility retardation pattern (Figure 2c). Interestingly, the same amount of HerPBK10 to neutralize a 5 kb plasmid also neutralizes a DNA ladder (Figure 2a). These results show that HerPBK10 can bind both double-stranded circular and linear DNA. To confirm that DNA binding occurs specifically through the polylysine sequence, non-polylysine tagged proteins, PB and Her, were incubated with the same 5 kb plasmid. On an agarose gel, plasmid incubated with HerPBK10 (Figure 2d, lane 2) or HerK10 (Figure 2d, lane 3) is retarded in mobility, whereas equivalent concentrations of Her (Figure 2d, lane 4) or PB (Figure 2d, lane 5) produce no such shift in mobility, thus establishing that DNA binding occurs predominantly through the polylysine domain (Figure 2d).

### *HerPBK10 binds heregulin receptors*

The human breast cancer cell line, MDA-MB-453, which overexpresses HER2-containing receptors, was used to test receptor binding of HerPBK10. Receptor binding activity was determined by an established assay that uses fluorescence activated cell sorting (FACS) to measure the amount of heregulin ligand required to displace the fluorescently tagged heregulin ligand, GFP-Her.[29] FACS analysis of MDA-MB-453 cells bound by GFP-Her are two orders of magnitude brighter than untreated cells (Figure 3a). A 10-fold higher concentration of non GFP-tagged Her reduces this fluorescence by nearly 95% (Figure 3c and g). The Ad5 knob protein, which we produced and purified from *E. coli* similar to our other recombinant proteins, is used here as a nonspecific competitor. A 10-fold higher concentration of the knob pro-

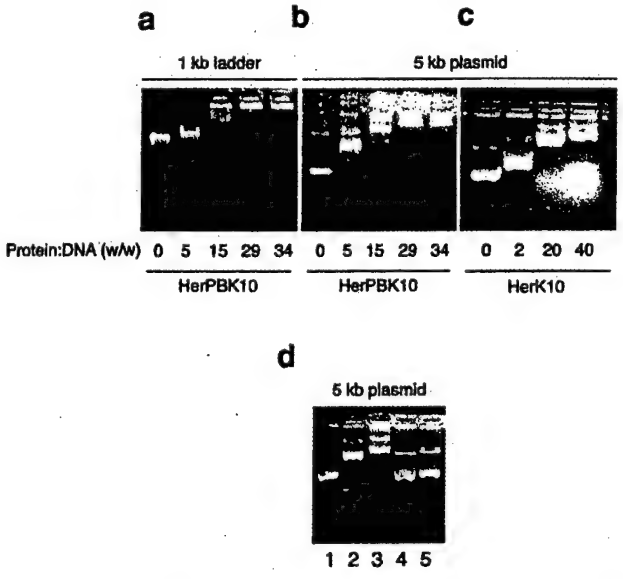


*Figure 2 DNA mobility shift analyses of lysine-tagged proteins. (a and b) HerPBK10 binds to linear and plasmid DNA. HerPBK10 was pre-incubated with (a) 200 ng of a 1 kb ladder whose sizes range from 75 bp to 12 kb, or (b) 200 ng of a 5 kb plasmid (pGFPemd-cmv) that is used in subsequent gene delivery assays. (c) HerK10 binds to DNA. HerK10 was pre-incubated with 500 ng of pGFPemd-cmv. (d) DNA binding occurs through the polylysine domain. The plasmid, pGFPemd-cmv (350 ng) was electrophoresed alone (lane 1) or after pre-incubation with 1 μmol concentrations of HerPBK10 (lane 2), HerK10 (lane 3), Her (lane 4), and PB (lane 5).*

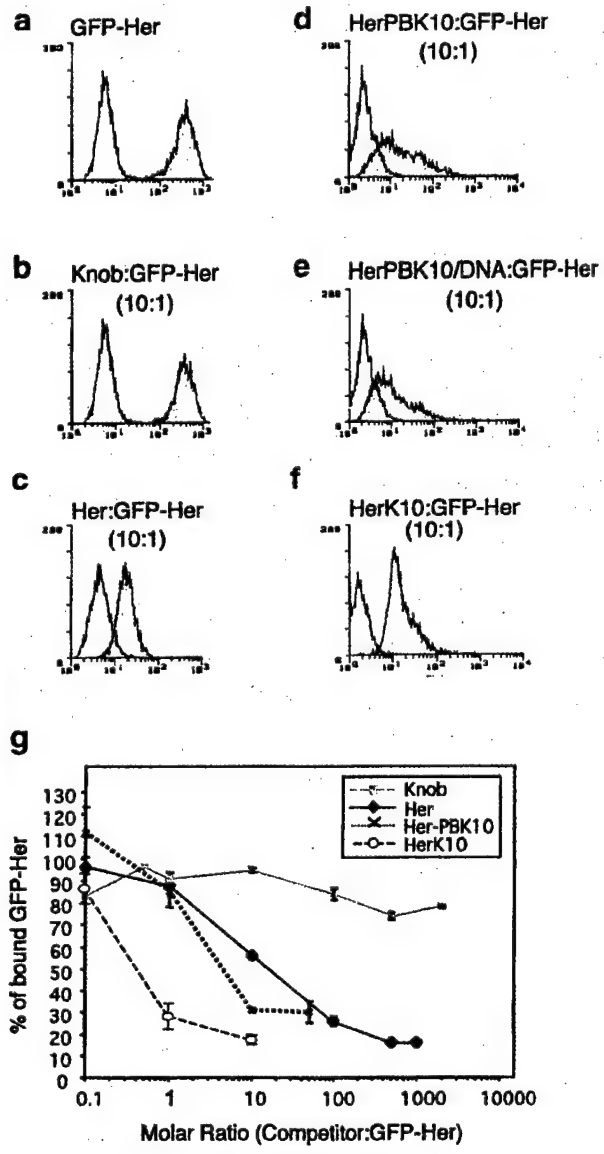


*Figure 3 Cell binding activity of recombinant proteins. MDA-MB-453 human breast cancer cells were incubated with GFP-Her (1 μM) alone (a) or with the indicated competitors (b–f), then quantified by FACS. The molar ratio of competitor to GFP-Her are shown in parentheses. Untreated and treated cell populations are shown by white and shaded histograms, respectively. (g) Summary of multiple FACS analyses. MDA-MB-453 cells were incubated with GPF-Her (0.1 μM) and increasing concentrations of either a nonspecific competitor (Knob, gray line), Her (black line), HerPBK10 (dotted line), or Her K10 (dashed line).*

tein has no effect on cell fluorescence, as previously established (Figure 3b and g). Increasing concentrations of HerPBK10[29] (Figure 3d and g) and HerK10 (Figure 3f and g) proteins, produce reductions in cell fluorescence similar to that produced by Her, as measured by FACS assay, suggesting that the receptor binding of HerPBK10 is unchanged from that of free heregulin ligand. In addition, the incubation of pGFPemd-cmv plasmid with HerPBK10 has no effect on the ability of HerPBK10 to compete away cell fluorescence (Figure 3e), indicating that DNA binding does not interfere with receptor binding activity.

### *Protamine protects HerPBK10/DNA complexes from serum protease degradation*

We have found previously that the addition of protamine to PBK10/DNA complexes protects the plasmid from serum nuclease activity and enhances gene delivery to cells in serum-containing culture medium without overriding the receptor-specific binding of the complexes.[12] To confirm the ability of protamine to protect HerPBK10-bound DNA, we incubated HerPBK10/DNA complexes formed at a standardized protein to DNA (w/w) ratio of 3 with increasing concentrations of protamine in the presence of 20% active serum. Complexes that are sensitive to nuclease activity are detected by the conversion of the plasmid from the supercoiled form to the open circle form, as described elsewhere.[32] In the presence of serum, nearly all of the plasmid DNA, whether in the presence or absence of HerPBK10, is converted to nicked forms by 30 min at 37°C (Figure 4). However, adding increasing concentrations of protamine preserves supercoiled DNA in the presence of active serum for up to 45 min (Figure 4). A protamine to DNA ratio (w/w) of 7 assures complete protection of the plasmid from nuclease-induced degradation.

### *HerPBK10 mediates gene delivery to breast cancer cells in culture*

To test the gene delivery capacity of complexes formed between HerPBK10, DNA and protamine, the pGFPemd-cmv reporter plasmid was used. We first performed these experiments in the absence of serum to determine the ratios of DNA to proteins required for optimal gene delivery. HerPBK10/DNA complexes formed at a protein to DNA (w/w) ratio of 3 were incubated with increasing concentrations of protamine as described earlier. MDA-MB-453 human breast cancer cells were exposed to these complexes and assayed 3 days after treatment for the expression of GFP.

In the absence of HerPBK10, transduction is nearly undetectable at all of the protamine concentrations that were tested (Figure 5a). In the presence of HerPBK10, increasing the concentration of protamine from 0 to 7 μg of protamine per μg of DNA enhances the green fluorescence from 0 to 6% GFP-positive cells (Figure 5a). A protamine to DNA w/w ratio greater than 7 reduces transduction to 3% GFP-positive cells, presumably by competing with HerPBK10 for DNA binding. Lipofectin, which was used as a nonspecific transduction control, produced 62 ± 2.6% GFP-positive cells. The maximum numbers of green fluorescent cells were detectable by 4 days after treatment (Figure 5a) and remained detectable up to 6 days after treatment (not shown). In subsequent assays, complexes were formed at a HerPBK10 to DNA (w/w) ratio of 3 and a protamine to DNA (w/w) ratio of 7, and will be referred to hereinafter as H2PO.

H2PO can also mediate the delivery and expression of a luciferase gene from the plasmid, pGL3 (Figure 6). Increasing the concentration of HerPBK10 from 0 to 5 ng in H2PO complexes containing pGL3 enhances the luminescence detected in transduced MDA-MB-453 cells by up to 34-fold over protamine alone. In the absence of HerPBK10, delivery of pGL3 by protamine alone produces negligible luciferase activity at all of the protamine concentrations that were tested. Cells treated with lipofectin produced almost eight times the luminescence of cells treated with H2PO (not shown).

We next examined the ability of H2PO to mediate similar levels of gene delivery in the presence of 1 and 10% serum, respectively. DNA complexed with protamine alone produces nearly undetectable gene delivery both in the absence and presence of serum (Figure 5b). H2PO enhances this delivery by nearly 20-fold in the absence of serum (Figure 5b). Importantly, similar levels of gene delivery by H2PO are observed in the presence of either 1 or 10% serum (Figure 5b). Thus, whereas the addition of protamine to the complex appears to mediate a protec-

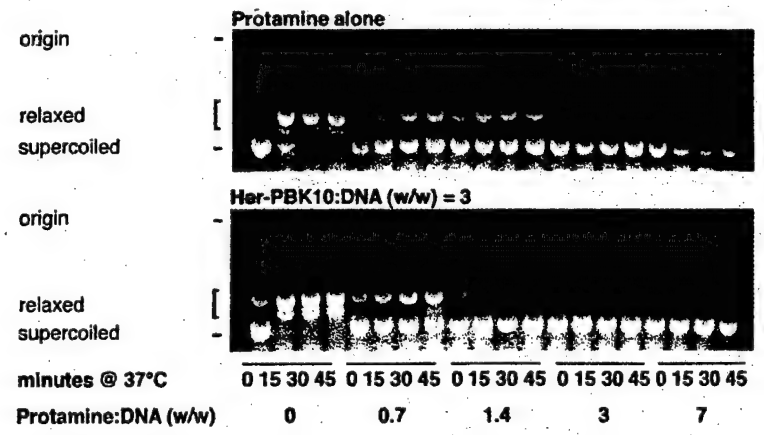


*Figure 4 DNA protection analysis. Plasmid DNA (pGFPemd-cmv, 350 ng) was mixed with protamine and/or HerPBK10 at the indicated ratios, incubated in 20% active (non-heat inactivated) fetal bovine serum, and electrophoresed at 50 V. The origin of electrophoresis, supercoiled DNA, and relaxed (nicked) plasmids are indicated.*

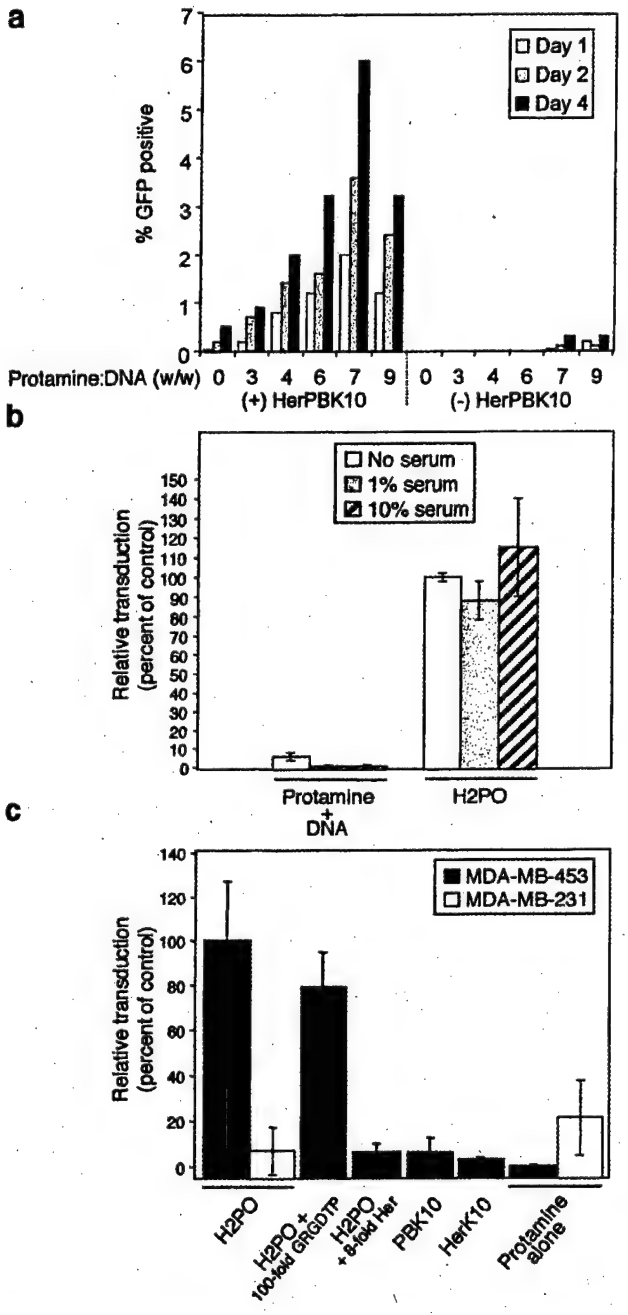


*Figure 5 Gene delivery to human breast cancer cells in culture. (a) HerPBK10 mediates gene delivery to MDA-MB-453 cells. Plasmid DNA (pGFPemd-cmv) was mixed with protamine and/or HerPBK10 at the indicated ratios and cells were assayed by detection of green fluorescence. Values are plotted as the percent of GFP positive cells. (b) Gene delivery to MDA-MB-453 cells in the presence of serum. Protamine was pre-incubated with the same plasmid used in H2PO complexes at a protamine to DNA (w/w) ratio of 7. Protamine/DNA complexes or H2PO were incubated with cells in either the absence or the presence of serum. In (b) and (c), transduction was determined by counting the percentage of GFP-positive cells of treated cell populations over untreated ones, and plotting the values as a percentage of H2PO-treated cells in the absence of serum. Error bars represent standard deviation. Experiments were performed in triplicate (c) Specificity of gene delivery by H2PO. All complexes contain a protamine to DNA (w/w) ratio of 7. H2PO, PBK10 and HerK10 complexes contain 1 μM HerPBK10, PBK10 and HerK10, respectively. The peptides GRGDTP and Her were incubated at a 100-fold and eight-fold molar excess, respectively, over HerPBK10.*

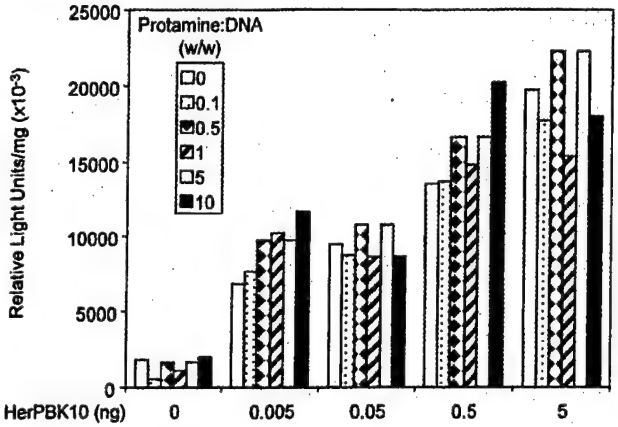


*Figure 6 H2PO-mediated delivery of a luciferase reporter gene to MDA-MB-453 cells in culture. Cells were seeded at a density of $5 \times 10^4$ cells/well of a 96-well dish and grown overnight. Cells were treated with H2PO complexes containing 0.1 μg pGL3 per well and the indicated concentrations of HerPBK10 and protamine, then assayed for luciferase activity 30 h later. Luciferase activity is expressed as relative light units (RLU)/mg of total protein.*

tive effect, HerPBK10 is still required for enhanced levels of gene delivery.

### *HerPBK10-mediated gene delivery is specific and requires the penton moiety*

To test the specificity of H2PO-mediated gene delivery, free heregulin protein (Her) was used as a competitor for heregulin receptor binding on MDA-MB-453 human breast cancer cells. In the presence of eight-fold higher concentrations of Her compared with HerPBK10, relative transduction (as measured by GFP fluorescence) is reduced by nearly 90% (Figure 5c). We also tested H2PO transduction of cells that do not express high levels of heregulin receptors. H2PO-mediated transduction of MDA-MB-231 cells, which express low to undetectable levels of HER-2 and -4,[31] produces only 12% of the GFP-fluorescence of MDA-MB-453 cells (Figure 5c). Together, these findings suggest that gene delivery by H2PO is mediated predominantly by specific binding to the heregulin receptor.

To determine whether integrins, the natural receptors for the penton protein, are also involved in mediating H2PO transduction, an RGD-containing peptide (GRGDTP) was used as a competitor for $\alpha_v$ integrin receptor binding. We have shown previously that similar complexes formed between PBK10, DNA and protamine (3PO) mediate gene delivery by integrin binding that is competitively inhibited by GRGDTP.[12] Interestingly, in this case, even at a concentration up to 100-fold higher than HerPBK10, GRGDTP produces no significant effect on H2PO-mediated gene delivery (Figure 5c).

Taken together, these findings suggest that cell binding by H2PO is not mediated by the integrin binding motif found on the penton moiety of HerPBK10, but rather by the heregulin receptor binding domain, Her. This also suggests that the requirement for integrin receptor-mediated endocytosis may be substituted by internalization via the heregulin receptor. The requirement for the heregulin domain to enhance targeted delivery is further demonstrated by comparison with the use of PBK10 as a targeting moiety in 3PO complexes exposed to MDA-MB-

453 cells. The delivery of the same concentration of a reporter plasmid to MDA-MB-453 cells by HerPBK10 exceeds that of PBK10 by more than 10-fold (Figure 5c).

The critical importance of the penton moiety is underscored when it is removed from the HerPBK10 construct to create the deletion mutant, HerK10. Whereas this protein binds DNA and heregulin receptors in a similar fashion to HerPBK10 (Figure 2c and Figure 3f and g), complexes made between HerK10, pGFPemd-cmv, and protamine produced 97% less green fluorescence than H2PO, and did not enhance transduction over protamine and DNA alone (Figure 5c). As competition for integrin binding by the RGD peptide did not appear to significantly affect overall transduction levels, it is plausible that the penton domain in HerPBK10 is required for the lysis of the endosomal vesicle after endocytosis.

To confirm whether H2PO is capable of endosome escape, H2PO was added to cells in the presence or absence of the acidotropic reagent, chloroquine. Although chloroquine inhibits endosome acidification, it also accumulates in intracellular vesicles, thereby inducing osmotic swelling and release of endosomal contents.[9,33,34] The presence of chloroquine has a notable effect on DNA delivery mediated by protamine alone, enhancing gene transfer nearly four-fold (Figure 7). In contrast, no effect is observed on H2PO-mediated gene transfer, which consistently maintained a nearly five-fold higher gene delivery over protamine alone.

## Discussion

We have demonstrated that the DNA/protein conjugate complex, H2PO, can be successfully targeted to breast cancer cells by means of the novel fusion protein, HerPBK10, which consists of a targeting ligand, an endosomolytic component and a DNA binding domain. Here, the receptor-binding motif of heregulin is used to mediate specific binding to breast cancer cells, and, as we have demonstrated previously, this ligand is rapidly endocytosed after cell binding. The penton protein used here does not participate in binding to an integrin receptor, but is essential, presumably, for lysis of the cellular endosome after internalization. Protamine protects the complexes from degradation by serum proteins. At the optimal ratios of protein to DNA, these complexes can transduce MDA-MB-453 human breast cancer cells *in vitro* by way of receptor-specific binding and internalization that is inhibited by free heregulin ligand. Accordingly, this method effectively targets gene delivery to cells expressing the receptor for heregulin.

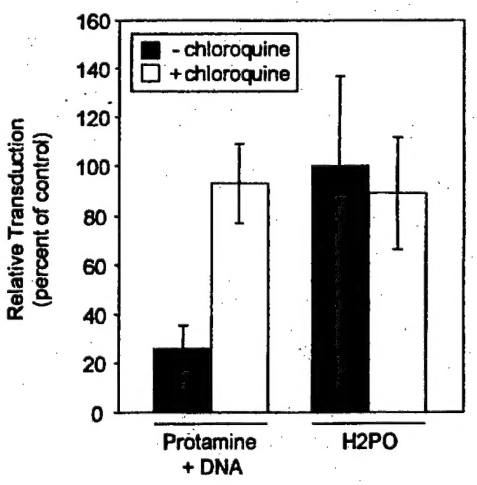


*Figure 7 Effect of chloroquine on gene delivery. MDA-MB-453 cells were treated either in the absence (filled bars) or presence (open bars) of a 100 μM final concentration of chloroquine. Relative transduction is expressed as a percentage of GFP-positive cells relative to that mediated by H2PO in the absence of chloroquine.*

This is the first demonstration, to our knowledge, of targeting the adenoviral penton protein for gene delivery by way of recombinant fusion to a receptor-specific ligand. There are few examples of similar systems for comparison. The closest in similarity is GD5, which incorporated domains for DNA binding, membrane translocation, and targeting to HER2-expressing cells.[34] The GD5 fusion protein contained a Gal4 DNA binding sequence, which required the presence of Gal4 *cis*-elements in the plasmid payload for complex formation. Targeting of this complex was accomplished by the inclusion of a single chain antibody fragment directed to the ErbB2 (HER2) subunit. The diptheria toxin (DT) membrane translocation domain was included for endosomal penetration. The GD5 fusion protein enhanced delivery of a luciferase reporter gene to HER2-containing cells by 25-fold over DNA complexes lacking the fusion protein. These data are in agreement with our observation of up to a 34-fold higher luciferase activity in cells targeted by H2PO, in comparison to complexes lacking HerPBK10.

The ability of the penton protein to mediate gene transfer across the endosome membrane has been established previously, suggesting that the penton is sufficient for receptor-mediated endocytosis and vesicle lysis.[12,35] The mechanism of endosome penetration, however, is still unclear. Studies of rhinovirus-mediated gene transfer suggest that viral fusogenic peptides form pores in the endosomal membrane in response to vesicle acidification, thus allowing the passage of endosomal contents into the cytosol.[36] Pore formation is the most plausible explanation for the membrane penetration activity of the DT translocation domain as well.[37] Lysis of the endosome by adenovirus, however, is likely due to a different mechanism since molecules of differing size are similarly released from endosomes *in vitro* by adenovirus, whereas the upper size exclusion by rhinovirus fusogenic peptides suggest the formation of pores of limiting size. This conceivably presents an advantage for gene transfer by penton proteins since they may override any size limitations imposed by channel-forming peptides.

A previous study has reported that the interaction of the penton protein with $\alpha_v\beta_5$ integrins is required for the promotion of endosomal penetration.[38] The data presented here suggest that integrin binding is not a determinant for penton-mediated gene transfer when the complexes are directed to a different receptor. Integrin-specific blocking peptides have a low to modest effect on gene delivery, suggesting that $\alpha_v$ integrins contribute minimally to the gene transfer process by H2PO. In contrast, the nearly complete abolition of delivery by free heregulin suggests that heregulin receptor binding and internalization is the predominant mode of cell entry by H2PO. In comparison to 3PO complexes, which are directed to integrin receptors, the incorporation of the heregulin ligand markedly improves delivery to MDA-MB-453 cells. This may be due to the higher affinity of

heregulin for its receptor in comparison to the affinity of the penton protein to $\alpha_v$ integrins, in addition to the amplification of the heregulin receptor subunits on MDA-MB-453 cells. Furthermore, we observed no increase in gene delivery by H2PO compared with protamine alone on MDA-MB-231 cells, which lack heregulin receptors, again supporting the important contribution of the heregulin moiety.

Although it is shown here that HerPBK10 exhibits saturable cell binding, H2PO produced 10% of the GFP-positive cells and 13% of the luciferase activity of transduction by the nonspecific gene transfer reagent, lipofectin. The endosomolytic agent, chloroquine, does not affect H2PO-mediated transduction, suggesting that the complexes are not trapped in endosomes after receptor-mediated endocytosis. A post-cytosolic entry step, therefore, may account for the observed difference between HerPBK10 receptor binding and final gene expression by H2PO.

We have thus demonstrated the feasibility of producing a chimeric penton protein for nonviral targeted gene delivery. As a protein-based gene delivery system, the HerPBK10 fusion protein presented here lends itself to the testing and development of improved functions for eventual gene therapy use, while avoiding the complications associated with producing targeted viral vectors. As each domain of the fusion protein can be conceived as a separate entity providing a distinct function to the overall molecular complex, modular replacement of the targeting ligand may be possible, thus producing novel proteins targeted to other cell types.

## Materials and methods

### *Cells, plasmids and peptides*

293 cells, HeLa cells and MDA-MB-453 human breast cancer cells were maintained in DMEM, 10% fetal bovine serum, at 37°C, 5% $CO_2$. The reporter plasmids, pGFPemd-cmv [R] control vector (Packard Instrument, Meriden, CT, USA), and pGL3 luciferase reporter vector (Promega, Madison, WI, USA) were used for gene delivery assays. The RGD-containing peptide (GRGDTP) was obtained from Sigma (St Louis, MO, USA).

### *DNA constructs*

A common 5′ oligonucleotide primer containing the sequence 5′-ATCGAAGGATCCATGCGGCGCGCGGCG ATGTAT-3′ was used to amplify both wild-type and lysine-tagged penton sequences from a pJM17 adenoviral genome template. The sequences of the 3′ primers are 5′-GCATCAGAATTCTCAAAAAGTGCGGCTCGATAG-3′ (PB) and 5′-CATGAATTCA$(TTT)_{10}$AAAAGTGCGGC TCGATAGGA-3′ (PBK10). A *Bam*HI restriction site was introduced in the 5′ primer and an *Eco*RI restriction site was introduced in the 3′ primers for in-frame insertion of both the wild-type and lysine-tagged pentons into the pRSET-A bacterial expression plasmid (Invitrogen, Carlsbad, CA, USA). This plasmid expresses the recombinant protein as an N-terminally histidine-tagged fusion for affinity purification by nickel chelate affinity chromatography. Polymerase chain reaction (PCR) amplification was used to add a sequence encoding a short polyglycine linker to the amino (N)-terminus of PBK10. The sequence encoding the linker contains a *Sac*II restriction site for additional cloning. The heregulin targeting ligand was produced by PCR amplification of the epidermal growth factor (EGF)-like domain of the heregulin gene[29] using a 5′ oligonucleotide primer containing a *Bam*HI site and a 3′ primer containing a *Sac*II site for cloning in-frame with PBK10. The targeting ligand was added to the lysine-tagged construct to create HerPBK10 by ligating the PCR product just N-terminal to PBK10. Construction of Her and GFP-Her are described elsewhere.[29] HerK10 was created by PCR amplification of the Her construct using the existing 5′ Her primer[29] and a 3′ oligonucleotide primer containing the sequence 5′-ATGAATTCA$(TTT)_{10}$AGA TCTACTTCCACCACTTCCACC-3′.

### *Protein expression and purification from bacteria*

Overnight cultures of BL21(DE3)pLysS (Novagen, Madison, WI, USA) bacterial transformants were inoculated 1:50 in LB containing 0.1 mg/ml ampicillin and 0.034 mg/ml chloramphenicol. When cultures reached an absorbance reading of ~0.6 at an optical density wavelength of 600 nm (OD 600), cultures were induced with 1 mM IPTG and grown for a further 4 h at 37°C with shaking. Cultures were harvested and pelleted. Cell pellets were resuspended in lysis buffer (50 mM Na-phosphate, pH 8.0, 500 mM NaCl, 5–10 mM imidazole, 1 mM phenylmethylsulfonyl fluoride) and lysed by addition of 0.1% Triton X-100 and one cycle of freeze–thawing. Supernatants were recovered, added to Ni-NTA resin (Qiagen, Valencia, CA, USA) pre-equilibrated in lysis buffer, and incubated for 1 h on ice. The resin containing bound protein was washed with 10 ml of lysis buffer, then 6 ml of a solution of 50 mM Na-phosphate, pH 8.0, 500 mM NaCl; 60 mM imidazole, and protein was eluted with 2 ml of a solution of 50 mM Na-phosphate, pH 8.0, 500 mM NaCl, 400 mM imidazole. Proteins were desalted on YM-10 nominal molecular weight limit spin columns (Millipore, Bedford, MA, USA) and their concentrations measured using the BioRad protein quantitation assay (BioRad Laboratories, Hercules, CA, USA).

### *Protein detection*

Denaturing polyacrylamide gel electrophoresis was performed in a discontinuous gel buffer system as previously described. Proteins were electrically transferred on to nitrocellulose using 39 mM glycine, 48 mM Tris-HCl, 0.0375% SDS, 20% methanol in a BioRad semi-dry transfer cell set at constant voltage (15 V) for 30 min. Blots were blocked with 3% milk in phosphate-buffered saline (PBS 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4$, 1.4 mM $KH_2PO_4$). Anti-His Tag antisera (Sigma) was used at a 1:3000 dilution in blocking buffer. Anti-Ad5 antisera (Access Bio Medical, San Diego, CA, USA) was used at a 1:8000 dilution in blocking buffer. Antibody–antigen complexes were detected by incubation with horseradish peroxidase (HRP) conjugated secondary antibodies (Sigma, St Louis, MO, USA), reaction with chemiluminescence detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA), and exposure to film (Hyperfilm ECL; Amersham Pharmacia Biotech).

### *Cell binding assay*

Cells were treated with GFP-tagged proteins as described elsewhere.[29] Briefly, MDA-MB-453 cells grown to 75% confluency were lifted with PBS supplemented with 2 mM EDTA, centrifuged, washed three to four times with

PBS, then added to PBS containing GFP-tagged proteins and 3% milk to reduce nonspecific binding. After a 1 h incubation on ice, cells were centrifuged, washed three to four times with PBS, resuspended in 0.5 ml PBS and measured by FACScan.

### *DNA mobility shift assays*

DNA was mixed with proteins at the indicated ratios in HEPES-buffered saline (HBS, 150 mM NaCl, 20 mM HEPES, pH 7.3) for 30 min at room temperature in a total volume of 10 μl. After incubation, 2 μl of sample dye was added to the mixtures, mixes were loaded on a 0.8% agarose gel, and electrophoresed at 50 V. Gels were stained with ethidium bromide after electrophoresis to visualize DNA bands. The 1 kb ladder was obtained from Life Technologies, Rockville, MD, USA.

### *DNA protection assay*

Plasmid and proteins were added together at various ratios in a total volume of 20 μl and incubated at room temperature for 30 min. Four μl of active (non-heat-inactivated) fetal bovine serum were added to each mix and mixtures were incubated at 37°C. SDS at a 1% final concentration was added to each mix to release the proteins from the DNA. Four μl of sample dye was added to each mix, and mixtures were electrophoresed on agarose gels as described earlier.

### *Cell delivery assays*

Cells were grown on 24-well dishes to approximately 75% confluency. Proteins and plasmids (3.5 μg) were incubated together at various ratios at room temperature for 30 min in 0.3 ml of adhesion buffer (DMEM, 2 mM $MgCl_2$, 20 mM HEPES), then added to cell monolayers. Cells were exposed to mixes for 3–5 h at 37°C, 5% $CO_2$, before the addition of complete serum. Cells were trypsinized and counted by FACS, where indicated, and using a hemocytometer under UV light microscopy to determine the percentage and absolute numbers of GFP-positive cells.

### *UV microscopy*

An Olympus IMT-2 inverted microscope fitted with an FITC filter was used to visualize GFP-positive cells. Photomicrographs were taken at 4× and 10× magnification.

### *FACS analysis*

Where indicated, 5000–10 000 events are counted on a Becton Dickinson FACScan analyzer (Becton Dickinson, Franklin Lakes, NJ, USA) using a 15 mW air-cooled argon laser set at 488 nm and recorded with a 530 nm emission filter in the FL1 emission channel. Cell populations are represented on a FACS histogram plotting green fluorescence intensity on a logarithmic scale against cell number. Fluorescence intensity of cell populations is indicated by a shift to the right of the histographical plots of treated cells. Fluorescence enhancement was determined by obtaining the number of gated fluorescent events for untreated and treated cells.

### *Luciferase assay*

Luciferase activity was determined using the Steady-Glo luciferase assay system (Promega) following the manufacturer's instructions. Briefly, to each well was added an equivalent volume of Steady-Glo reagent. The dish was incubated at room temperature for 5 min to allow lysis, and wells were read directly at 5 s per well using an MLX microtiter plate luminometer (Dynex Technologies, Chantilly, VA, USA) using the Glow Endpoint assay type set on autogain. Protein concentrations were determined from aliquots of lysed samples using the BioRad protein quantification assay (BioRad Laboratories).

## Acknowledgements

We are grateful to the following people for ongoing discussions and support: Xinhua Chen, Gene Chung, Yasuo Hamamori, John Hwang, Tatsuya Iso, Kimi Kong, Vivian Leung, Jochen Muller-Ehmsen, Alex Oxyzoglou, Coralie Poizat, Tsuyoshi Sakoda, Vittorio Sartorelli, Terry Saluna, David Tinsley and Hung-Yi Wu. Grants to LHK from the Susan G Komen Breast Cancer foundation, the Department of Defense, and the National Institutes of Health (CA59318-07) and a fellowship to LKM-K from the National Institutes of Health (HL09638-02) supported this work.

## References

1 Ilan Y *et al*. Oral tolerization to adenoviral antigens permits long-term gene expression using recombinant adenoviral vectors. *J Clin Invest* 1997; **99**: 1098–1106.
2 Yang Y, Ertl HC, Wilson JM. MHC class I-restricted cytotoxic T lymphocytes to viral antigens destroy hepatocytes in mice infected with E1-deleted recombinant adenoviruses. *Immunity* 1994; **1**: 433–442.
3 Kozarsky KF, Wilson JM. Gene therapy: adenovirus vectors. *Curr Opin Genet Dev* 1993; **3**: 499–503.
4 Greber UF, Willets M, Webster P, Helenius A. Stepwise dismantling of adenovirus 2 during entry into cells. *Cell* 1993; **75**: 477–486.
5 Bergelson JM *et al*. Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. *Science* 1997; **275**: 1320–1323.
6 Wickham TJ, Mathias P, Cheresh DA, Nemerow GR. Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ promote adenovirus internalization but not virus attachment. *Cell* 1993; **73**: 309–319.
7 Seth P *et al*. Evidence that the penton base of adenovirus is involved in potentiation of toxicity of Pseudomonas exotoxin conjugated to epidermal growth factor. *Molec Cell Biol* 1984; **4**: 1528–1533.
8 Karayan L *et al*. Structural and functional determinants in adenovirus type 2 penton base recombinant protein. *J Virol* 1997; **71**: 8678–8689.
9 Fominaya J, Wels W. Target cell-specific DNA transfer mediated by a chimeric multidomain protein. Novel non-viral gene delivery system. *J Biol Chem* 1996; **271**: 10560–10568.
10 Gottschalk S *et al*. A novel DNA-peptide complex for efficient gene transfer and expression in mammalian cells. *Gene Therapy* 1996; **3**: 48–57.
11 Fisher KJ, Wilson JM. The transmembrane domain of diphtheria toxin improves molecular conjugate gene transfer. *Biochem J* 1997; **321**: 49–58.
12 Medina-Kauwe LK, Kasahara N, Kedes L. 3PO, a novel nonviral gene delivery system using engineered Ad5 penton proteins. *Gene Therapy* 2001; **8**: 795–803.
13 Bacus SS, Zelnick CR, Plowman G, Yarden Y. Expression of the erbB-2 family of growth factor receptors and their ligands in breast cancers. Implication for tumor biology and clinical behavior. *Am J Clin Pathol* 1994; **102**: S13–S24.
14 Carraway III KL, Cantley LC. A neu acquaintance for erbB3 and erbB4: a role for receptor heterodimerization in growth signaling. Cell 1994; 78: 5–8.
15 Carraway III KL et al. The erbB3 gene product is a receptor for heregulin. J Biol Chem 1994; 269: 14303–14306.

16 Carraway III KL, Burden SJ. Neuregulins and their receptors. Curr Opin Neurobiol 1995; 5: 606–612.
17 Goldman R, Levy RB, Peles E, Yarden Y. Heterodimerization of the erbB-1 and erbB-2 receptors in human breast carcinoma cells: a mechanism for receptor transregulation. *Biochemistry* 1990; **29**: 11024–11028.
18 Hung MC *et al*. HER-2/neu-targeting gene therapy - a review. *Gene* 1995; **159**: 65–71.
19 Press MF *et al*. HER-2/neu oncogene amplification and expression in breast and ovarian cancers. *Prog Clin Biol Res* 1990; **354A**: 209–221.
20 Slamon DJ *et al*. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 1987; **235**: 177–182.
21 Slamon DJ, Clark GM. Amplification of c-erbB-2 and aggressive human breast tumors?. *Science* 1988; **240**: 1795–1798.
22 Yarden Y, Weinberg RA. Experimental approaches to hypothetical hormones: detection of a candidate ligand of the neu protooncogene. *Proc Natl Acad Sci USA* 1989; **86**: 3179–3183.
23 Holmes WE *et al*. Identification of heregulin, a specific activator of p185erbB2. *Science* 1992; **256**: 1205–1210.
24 Lenferink AE *et al*. Differential endocytic routing of homo- and hetero-dimeric ErbB tyrosine kinases confers signaling superiority to receptor heterodimers. *EMBO J* 1998; **17**: 3385–3397.
25 Li W *et al*. Heregulin is rapidly translocated to the nucleus and its transport is correlated with c-myc induction in breast cancer cells. *Oncogene* 1996; **12**: 2473–2477.
26 Sliwkowski MX *et al*. Coexpression of erbB2 and erbB3 proteins reconstitutes a high affinity receptor for heregulin. *J Biol Chem* 1994; **269**: 14661–14665.
27 Tzahar E *et al*. ErbB-3 and ErbB-4 function as the respective low and high affinity receptors of all Neu differentiation factor/heregulin isoforms. *J Biol Chem* 1994; **269**: 25226–25233.
28 Waterman H, Sabanai I, Geiger B, Yarden Y. Alternative intracellular routing of ErbB receptors may determine signaling potency. *J Biol Chem* 1998; **273**: 13819–13827.
29 Medina-Kauwe LK, Leung V, Wu L, Kedes L. Assessing the binding and endocytosis activity of cellular receptors using GFP-ligand fusions. *BioTechniques* 2000; **29**: 602–609.
30 Harris CE *et al*. Receptor-mediated gene transfer to airway epithelial cells in primary culture. *Am J Resp Cell Molec Biol* 1993; **9**: 441–447.
31 Han X, Kasahara N, Kan YW. Ligand-directed retroviral targeting of human breast cancer cells. *Proc Natl Acad Sci USA* 1995; **92**: 9747–9751.
32 Gao X, Huang L. Potentiation of cationic liposome-mediated gene delivery by polycations. *Biochemistry* 1996; **35**: 1027–1036.
33 Fominaya J, Uherek C, Wels W. A chimeric fusion protein containing transforming growth factor-α mediates gene transfer via binding to the EGF receptor. *Gene Therapy* 1998; **5**: 521–530.
34 Uherek C, Fominaya J, Wels W. A modular DNA carrier protein based on the structure of diphtheria toxin mediates target cell-specific gene delivery. *J Biol Chem* 1998; **273**: 8835–8841.
35 Fender P *et al*. Adenovirus dodecahedron, a new vector for human gene transfer. *Nature Biotechnol* 1997; **15**: 52–56.
36 Prchla E *et al*. Virus-mediated release of endosomal content *in vitro*: different behavior of adenovirus and rhinovirus serotype 2. *J Cell Biol* 1995; **131**: 111–123.
37 O'Keefe DO *et al*. pH-dependent insertion of proteins into membranes: B-chain mutation of diphtheria toxin that inhibits membrane translocation, Glu-349-Lys. *Proc Natl Acad Sci USA* 1992; **89**: 6202–6206.
38 Wickham TJ, Filardo EJ, Cheresh DA, Nemerow GR. Integrin alpha v beta 5 selectively promotes adenovirus mediated cell membrane permeabilization. *J Cell Biol* 1994; **127**: 257–264.